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Regulation of the mTOR/S6K pathway by cellular energy

INAUGURALDISSERTATION

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Adem Kalender
aus Brüssel, Belgien

Cincinnati, 2009

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag der Herren Professoren M. Hall, G. Thomas und M. Wymann

Basel, den 13. November 2007

Prof. Dr. Hans-Peter Hauri, Dekan

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ACKNOWLEDGEMENTS

I would like to warmly thank Dr. George Thomas and Dr. Sara Kozma for hosting me in their laboratory and for giving me the opportunity to carry out my PhD research thesis in such privileged laboratory conditions.

I would also like to thank my faculty representative Prof. Michael Hall for his support during my thesis and my co-referee Prof. Matthias Wymann for his time and his patience.

I am immensely indebted to Dr. Anand Selvaraj for his constant support, never failing to help and challenge me. His enthusiasm and his way of approaching science were very influential. His patience and friendship will always be acknowledged and appreciated.

I am also very thankful to Dr. Stefano Fumagalli for being a buddy in this Cincinnati adventure. Your friendship, support and help have played an important part.

I would like to thank Dr. Joshi Venugopal for his immense patience and friendship all along this pain. For all the good and bad times we lived together and to future meetings.

To past members of the Thomas lab, namely Alessandro Di Cara, So Young Kim, Jacques Montagne, Gael Gangloff and Thomas Radimerski for teaching me a lot of useful tips, but also to the Cincinnati crowd, Pat Dennis, Marie-Laure Ruhf, Akiko Kuma, Larissa Carnevalli, Takahiro Nobukini and everybody else in the lab for their help and good spirit.

I would like to thank all my family for their constant love and support, especially my mum and dad despite the long distance and the few occasions that we managed to see each other for all this time. You have a lot to be proud of and I deeply thank you for your patience and understanding all along. Last but not least, all this time and despite the distance and the time we missed, you never faded away. Thank you for being my friend.

ABBREVIATIONS

$\Delta\Psi_m$	Mitochondrial membrane potential
2DG	2-deoxy-α-D-glucose
4E-BP1	Eukaryotic translation initiation factor 4E (eIF4E) binding protein 1
ACC	Acetyl-CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AMPK	AMP-dependent protein kinase
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
dsRNAi	Double stranded RNA interference
ELB	Egg lysis buffer
GAP	GTPase-activating protein
h	Hours
HKII	Hexokinase II
HPLC	High performance liquid chromatography
IRS	Insulin receptor substrate
MEF	Mouse embryonic fibroblast
min	Minutes
mTOR	Mammalian target of rapamycin
PAGE	Polyacrylamide gel electrophoresis
PAO	Oxidant phenylarsine oxide
PBS	Phosphate-buffered saline
PI(3)P	Phophatidylinositol 3-phosphate
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RT	Room temperature
S6K1	p70/p85 S6 kinase
TBST	Tris-buffered saline with 0.1% Tween-20 detergent
TMRE	Tetramethylrhodamine ethyl ester
TSC	Tuberous sclerosis complex

I. ABSTRACT

The mammalian target of rapamycin (mTOR) signaling pathway integrates positive and negative signals that control cellular growth, metabolism and survival. mTOR exists in two different complexes, mTOR Complex1 and mTOR Complex2. mTOR Complex1, which is rapamycin-sensitive, phosphorylates ribosomal S6 kinase 1 (S6K1) and initiation factor 4E binding proteins (4E-BPs). mTOR Complex2, which is rapamycin-insensitive, phosphorylates and activates protein kinase B (PKB/Akt). Both mTOR complexes are stimulated by mitogens, but only mTOR Complex1 is under the control of nutrients and cellular energy status. With respect to cellular energy status, mTOR Complex1 signaling is sensitive to inhibition of both glycolytic flux and mitochondrial oxidative phosphorylation. In brief, energy deprivation affects mTOR Complex1 through two routes: an acute rapid response and a chronic long lasting response. Here we describe the mechanisms by which energy depletion influences mTOR Complex1 signaling, largely focusing on the acute response. Previous studies, mainly based on correlative evidence, argued that the acute energy deprivation response is mediated by adenosine mono phosphate-dependent protein kinase (AMPK) through the activation of the tumor suppressor, Tuberous Sclerosis Complex 1 and 2 (TSC1/2). We used specific knockout cell lines to address this issue and, unexpectedly, found that TSC1/2, recognized as a point of convergence for a number of specific signals, is dispensable for the regulation of mTOR Complex1 by acute energy depletion. Strikingly, neither the inhibitory acute nor the chronic energy-deprivation response towards mTOR Complex1 requires AMPK. Moreover, the upstream activator of AMPK, the serine/threonine protein kinase 11 (STK11/LKB1) is also dispensable for the acute energy depletion response to mTOR Complex1 signaling. The results demonstrate that acute energy depletion signals operate independently of the LKB1-AMPK-TSC2 axis on mTOR Complex1, revealing a novel autonomous energy-dependent mTOR Complex1 signaling pathway. Importantly, we find that metformin, a widely prescribed drug for the treatment of *diabetes mellitus* type II, which is thought to operate through the LKB1-AMPK-TSC2 axis, affects mTOR Complex1 signaling through this same autonomous energy-dependent pathway, independent of AMPK and TSC. The significance of these findings is underscored by recent clinical studies showing that patients using metformin have a lower incidence of tumor development.

II.INTRODUCTION

1. Glycolysis and oxidative phosphorylation

Adenosine 5'-triphosphate (ATP), required for normal cell proliferation and survival, comes primarily from two sources: glycolysis and the tricarboxylic acid (TCA) or Krebs cycle. Glycolysis is a series of metabolic processes by which one molecule of glucose is catabolized into two molecules of pyruvate in the cytoplasm to produce a net gain of 2 ATPs from each molecule of glucose (Figure 1). It can be summarized as the following overall reaction:

$$\mathbf{Glucose + 2P_i + 2ADP + 2NAD^+ \rightarrow 2\ Pyruvate + 2ATP + 2NADH + 2H^+ + 2H_2O}$$

The glycolytic pathway has two phases, the priming phase and the energy-yielding phase. The priming phase uses two molecules of ATP to convert glucose to fructose-1,6-bisphosphate through sequential reactions catalysed by hexokinase, phosphoglucose isomerase, and phosphofructokinase. In the second phase, fructose-1,6-bisphosphate is further converted stepwise into pyruvate with the production of four molecules of ATP and two molecules of nicotinamide adenine dinucleotide hydrogen (NADH). During this process, two ATP and two NAD^+ are consumed. In the absence of oxygen, NAD^+ is regenerated from NADH by reduction of pyruvate to lactic acid catalysed by lactate dehydrogenase (Figure 1). Under aerobic conditions, pyruvate can then be further oxidized to carbon dioxide (CO_2) and water (H_2O) in the mitochondria through the TCA cycle and the respiratory chain, yielding a large amount of ATP. Each reaction in the glycolytic pathway is catalysed by a specific enzyme or enzyme complex. However, in addition to their well-characterized enzymatic activities recent studies suggest that some of the glycolytic enzymes are multi-functional proteins involved in additional important cellular processes including the regulation of transcription and apoptosis. Furthermore, although glycolysis is the classical metabolic pathway that generates pyruvate, glyceraldehyde-3-phosphate can also be produced by the pentose phosphate pathway (PPP) therefore joining the second phase of the glycolytic pathway yielding pyruvate and ATP. Pyruvate formed from glycolysis is converted in a series of reactions in the Krebs cycle that donates electrons via NADH and flavin adenine

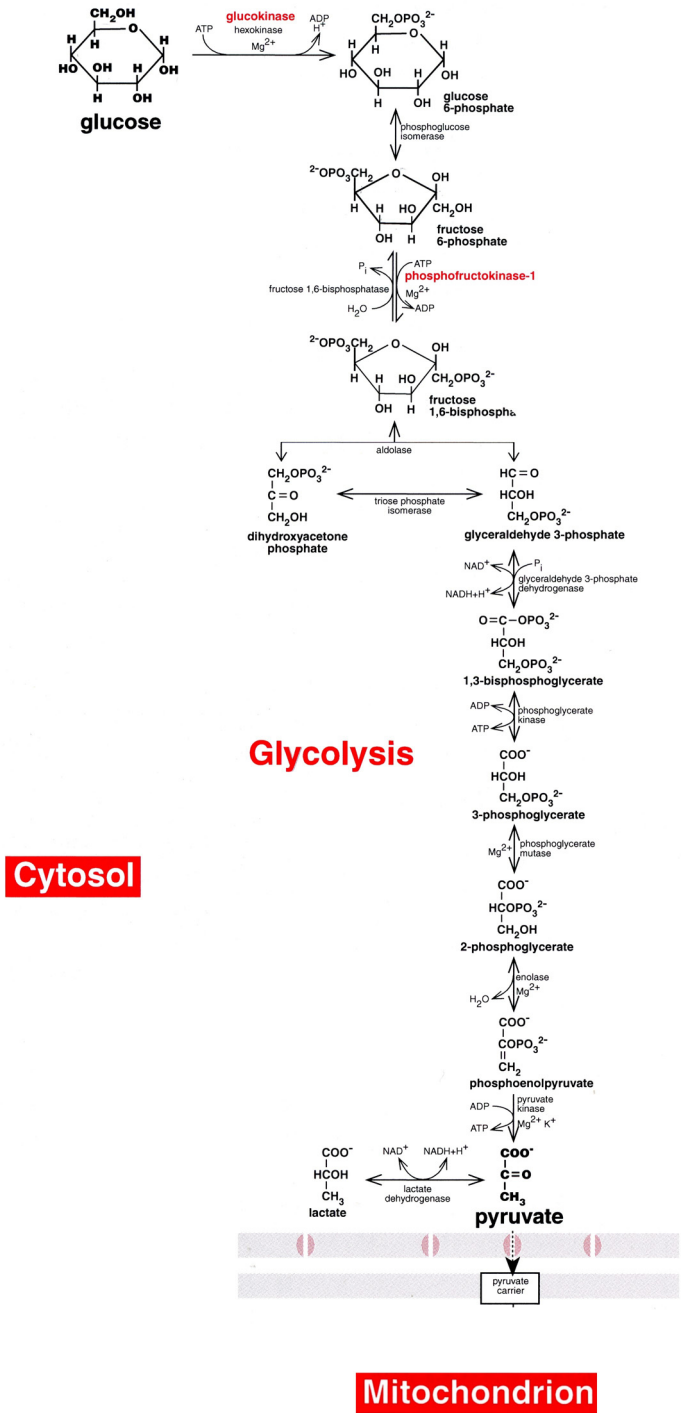


Figure 1: The glycolytic pathway (from Metabolism at a glance, reproduced with permission from Blackwell Publishing)

dinucleotide dihydrogen ($FADH_2$) to the respiratory chain complexes in mitochondria. The electrons are passed on within the respiratory chain to generate an electrochemical gradient across the inner mitochondrial membrane; this proton gradient is termed mitochondrial membrane potential ($\Delta\Psi_m$) (See Figures 2 and 3). With oxygen serving as the final electron acceptor to form water, the proton gradient provides the energy necessary for the F_0F_1 ATPase complex or ATP synthetase to generate ATP from adenosine 5'-diphosphate (ADP) and inorganic phosphate ions (P_i), a process called oxidative phosphorylation (OXPHOS) (See Figures 2 and 3). This process will yield 36 molecules of ATP per molecule of glucose. Under conditions of limited oxygen, such as muscle that has undergone prolonged exercise, pyruvate is not used in the TCA cycle and is converted into lactic acid by lactate dehydrogenase (LDH) in a process termed anaerobic glycolysis.

2. Bio-energetic in the cancer cell

Many cancer cells consume glucose avidly and produce lactic acid rather than catabolizing glucose via the TCA cycle, which is a key step for generating ATP in nonhypoxic normal cells. The upregulation of glycolysis and the shift toward lactate production in cancers, even in the presence of adequate oxygen, is termed the Warburg effect or aerobic glycolysis (Warburg 1956). Tumors display aerobic glycolysis partly through activation of oncogenes or loss of tumor suppressors, which are then further enhanced by stabilization of the hypoxia inducible factor 1 (HIF-1). This is brought about via an adaptive response to a hypoxic microenvironment or through pathways that stabilize HIF under nonhypoxic conditions. The stabilization of HIF-1 is thought to be the prime driving mechanism for enhanced glycolysis observed in tumours due to activation of the transcription and translation of glycolytic genes. HIF-1 is a transcription factor constituted by two subunits, HIF-1α and HIF-1β. Factor stability mostly depends on HIF-1α. In aerobic conditions, an active process of HIF-1α degradation is promoted, whereas in anaerobiosis, HIF-1α becomes stable (Semenza 2000) (Guppy, Leedman et al. 2002). In addition to hypoxia, HIF-1α may be induced, under aerobiosis, by cytokines, growth factors, reactive oxygen species (ROS) or by the energy-metabolism intermediates

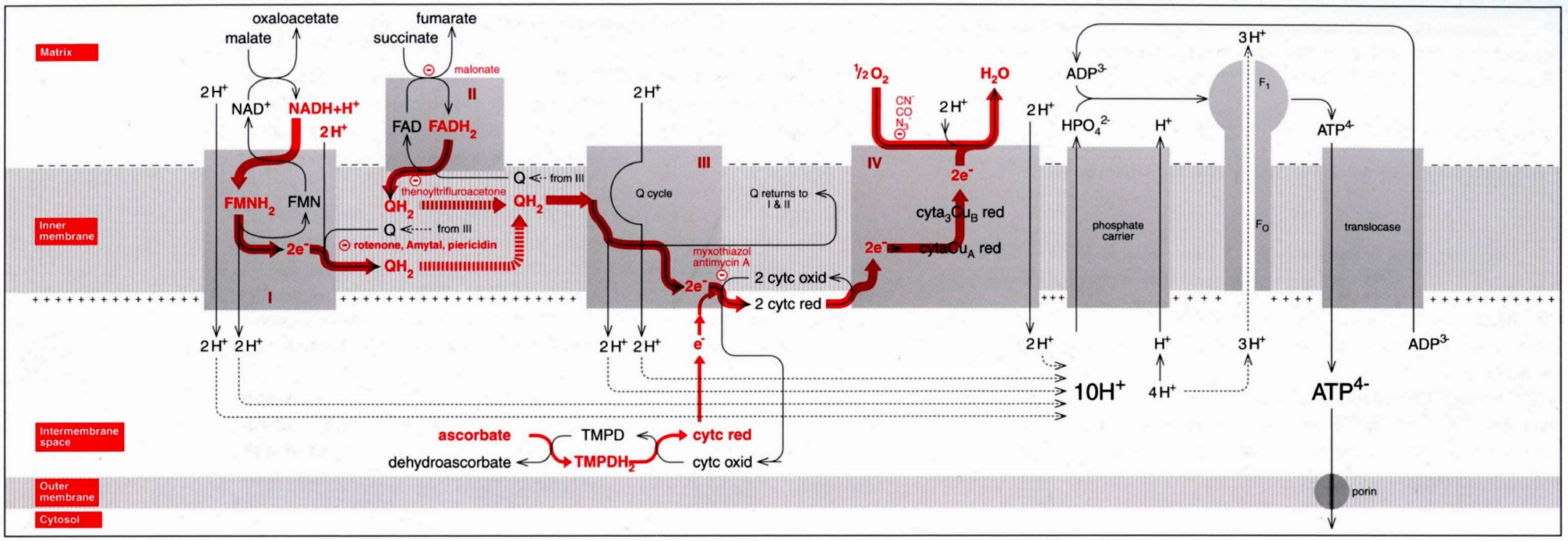


Figure 2: The electron transport - the respiratory chain showing the flow of electrons from NADH and $FADH_2$ to oxygen with the formation of water (from Metabolism at a glance, reproduced with permission from Blackwell Publishing)

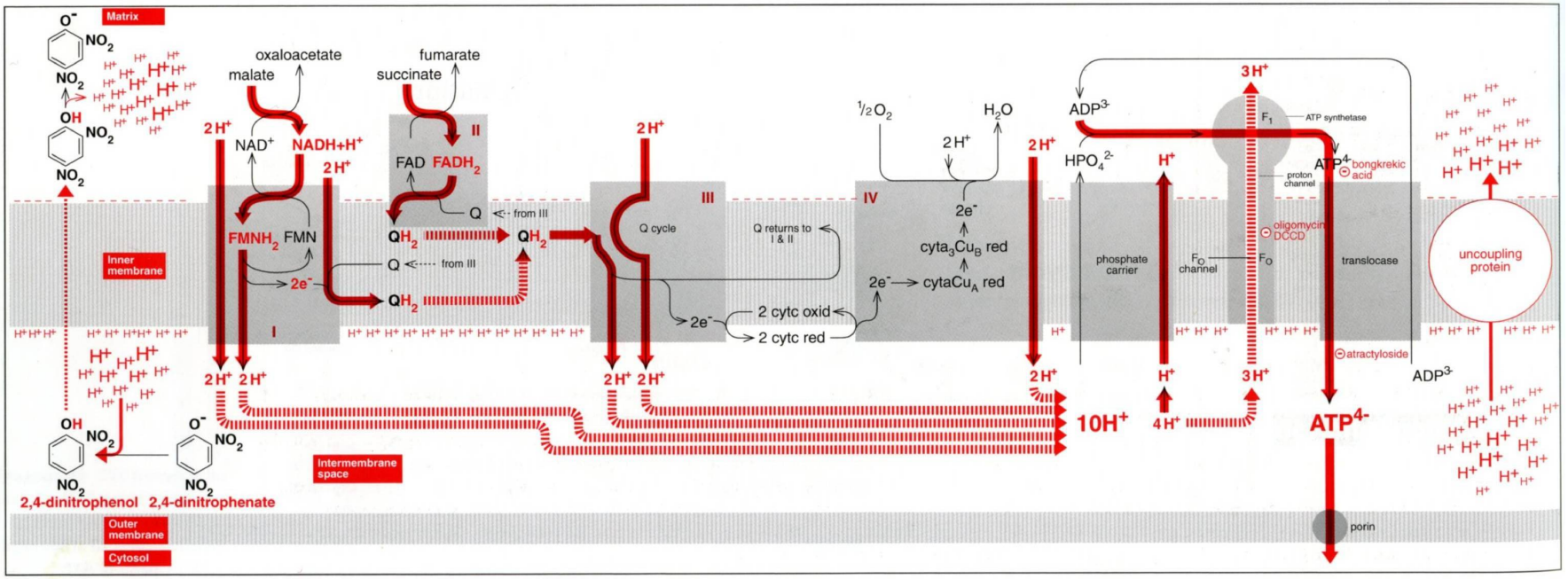


Figure 3: The proton flow – the respiratory chain showing the extrusion of protons by complexes I, III and IV creating an electrochemical gradient. As the protons return through the ATP synthetase complex, ADP is phosphorylated to ATP (from Metabolism at a glance, reproduced with permission from Blackwell Publishing)

pyruvate, lactate and oxaloacetate (Guppy, Leedman et al. 2002) (Dalgard, Lu et al. 2004). The von Hippel-Lindau protein, a tumor suppressor, binds to HIF-1α and induces its degradation by the proteosome. In some aggressive tumors, the von Hippel-Lindau protein is mutated, thus becoming ineffective in promoting HIF-1α degradation. This might be the reason why HIF-1α is only detected in malignant tumors, but not in normal, healthy tissues or benign tumors (Guppy, Leedman et al. 2002; Robey, Lien et al. 2005). In turn, HIF-1α stabilization promotes the expression of various glycolytic genes such as hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase and LDH and others (Dang and Semenza 1999), leading to increased glycolytic flux. Notwithstanding the O_2 level, metastatic tumor cell lines (breast MDA, U87 glioblastoma, renal RCC4) show high levels of HIF-1α, over-expression of glycolytic enzymes and high rates of glycolysis, whereas in nonmetastatic tumor cells (breast MCF-7, HT-29 colon, A549 lung) increased HIF-1α, enzyme over-expression and glycolysis are only observed under conditions of hypoxia (Robey, Lien et al. 2005). Separately, the oncogene, c-myc, a transcription factor, may also activate glycolytic genes in transformed cells, such as glucose transporter 1 (GLUT1), hexose-6-phosphate isomerase, PFK-1, GAPDH, phosphoglycerate kinase, LDH, increasing glycolysis under conditions of aerobiosis (Dang, Lewis et al. 1997) (Osthus, Shim et al. 2000).

One of the most critical and rate limiting steps in glycolysis is the ATP-dependent phosphorylation of glucose to form glucose-6-phosphate through the catalysis of tissue-specific isoenzymes known as hexokinases (HK) (Mathupala, Ko et al. 2006). This phosphorylation converts the nonionic glucose to an anion that is trapped in the cells, glucose-6-phosphate. This molecule serves as the starting point for glucose to enter the glycolytic pathway, the pentose phosphate pathway or glycogen synthesis pathway. In mammalian cells there are four different isoforms of HK (HK-I, -II, -III, and -IV, or glucokinase), which differ in their enzymatic properties as well as in their tissue-specific expression and subcellular localization (Wilson 2003). The predominant isoform in brain, mammary gland, kidney and retina is HK-I (Wilson 2003). HK-II predominates in skeletal muscle and adipose cells, although its activity is relatively low (Pedersen, Mathupala et al. 2002). Interestingly, the expression of these isoenzymes is different between cancer cells and non-transormed cells, with cancer cells generally exhibiting an especially high concentration of HK-II, except for brain tumors in which HK-I is the over-expressed isoform (Pedersen, Mathupala et al. 2002; Wilson 2003). Because they contain a

specific hydrophobic N-terminal segment, HK-I and HK-II may be either bound to the outer mitochondrial membrane or free in the cytosol (Pedersen, Mathupala et al. 2002). In fast-growing tumor cells, HK-II has been found to have enhanced binding to the outer mitochondrial membrane (da-Silva, Gomez-Puyou et al. 2004). The apparent specific site of HK-II binding to the outer membrane is the voltage-dependent anion channel (VDAC) or porin (Beutner, Ruck et al. 1996). Such interaction protect HK-II from proteases and provides direct access to the newly synthesized ATP by the ATP synthetase. The fact that oxidative phosphorylation might be efficiently coupled to the glycolytic pathway via the mitochondrial-bound hexokinase would give cancer cells a clear growth advantage. The other rate limiting step in glycolysis is attributed to phosphofructokinase, which catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, using ATP as the energy source. Three forms of phosphofructokinase, M (muscle), L (liver), and P (platelet), have been identified in humans (Dunaway, Kasten et al. 1988). Phosphofructokinase is allosterically regulated by 2,3-diphosphoglycerate (Layzer, Rowland et al. 1969). However, other allosteric regulators include fructose 2, 6 bisposphate, phosphenolpyruvate and AMP. Although its implication in cancer is unclear, inhibition by phosphenolpyruvate is thought to increase the glycolytic flux (Sanchez-Martinez, Estevez et al. 2000).

Glucose uptake in mammalian tissues is achieved by a set of five transmembrane transporters termed Glut (glucose transporter) 1–5, which are encoded by different Glut genes (Medina and Owen 2002). Similar to the HK isoforms, the Glut isoforms also differ in their transport kinetics. Increased glucose transport in malignant tumors has been associated with increased and deregulated expression of these transporters, mostly with over-expression of the Glut-1 isoform. In human tumors, a high level of Glut-1 expression has been associated with poor prognosis (Macheda, Rogers et al. 2005). As Glut expression at the cell-surface is mediated by hormone-induced cycling of transporter vesicles between intracellular pools and the cell membrane, deregulated trafficking may contribute also to an enhanced display of Glut on malignant tumors thus facilitating enhanced glucose uptake (Smith 1999). Moreover, the role of Gluts in highly malignant tumors is still an area of intense investigation, relating to the mechanism by which key transporters are involved in delivering glucose to HK II bound to VDAC of the mitochondrial outer membrane. Current knowledge suggests that glucose must diffuse through quite a distance from Glut transporter to HK II, which probably represents an unlikely scenario. Considering that highly malignant cancer cells

and the mitochondria within them are not static, but likely dynamic, it does not seem unreasonable to suggest that the Glut on the cell membrane and HK II bound to VDAC on mitochondria come into contact. However, it is clear that other factors and enzymes are implicated in the glycolytic phenotype of cancer cells. Nevertheless, it is becoming apparent that the switch to glycolytic metabolism may contribute to tumor development through enhanced glycolytic flux and/or the multifaceted functions of glycolytic enzymes. It should be noted, however, that the role of normal mitochondrial function in tumorigenesis is not well defined; therefore a deeper understanding of mitochondrial function in cancer glucose metabolism would uncover additional layers of players and regulations of energy metabolism.

3. Energy homeostasis

A fundamental principle in multicellular organisms is the strict maintenance of stable concentrations of intracellular oxygen and ATP, the universal energy currency of biological systems. Upon activation of excitable cells, such as skeletal and cardiac muscle, or brain and nerve cells, ATP turnover rates may increase by several orders of magnitude within seconds, but the concentration of ATP remains remarkably stable and ATP:ADP ratios, as well as ATP:AMP ratios (see below), are maintained as high as possible to guarantee optimal efficiency for cellular ATPases that are at work to perform a multitude of energy-dependent cellular activities, such as muscle contraction, cell motility and ion pumping (Wallimann 1994). ATP homeostasis and maintenance of high ATP:ADP and ATP:AMP ratios are facilitated by the action of two well known enzyme systems, working fast and efficient energy safeguards. First, creatine kinase (CK), efficiently regenerating ATP at the expense of phosphocreatine (PCr) by the following reaction:

$$\mathbf{PCr + ADP \rightarrow ATP + Cr}$$

where Cr is creatine. Second, is through adenylate kinase (AK), which converts two ADP molecules into one ATP and one AMP. These two enzymes working together in an intricate subcellular energy distribution network or circuit (Wallimann, Wyss et al. 1992) (Dzeja, Zeleznikar et al. 1998), temporally and, due to their subcellular

microcompartmentation, spatially buffer subcellular ATP levels (Bessman and Geiger 1981) (Ventura-Clapier, Kuznetsov et al. 1998). A low cellular PCr:ATP ratio, indicating a low energy state cells and tissue, are observed in pathological states like cardiac insufficiency and many neurodegenerative diseases (Nascimben, Ingwall et al. 1996). However, an important question is when cells are energetically compromised, for instance during nutritional deprivation, oxidative damage, heat shock and an ischaemic insult, how are they able to sense their low energy status? Moreover, what are the mechanisms by which cells adapt to an energy crisis to induce compensatory or protective pathways through re-programming its gene transcription machinery? For instance, it has been shown that in the case of a fast-twitch glycolytic skeletal muscle, which is rich in PCr, the energy demand is accomplished first by using the PCr reserves in order to keep the ATP levels constant in the cell. Once the PCr levels decline to about 70–80%, the ATP concentration starts to fall, which coincides with the appearance of ADP, and then later AMP (McGilvery and Murray 1974). Therefore, the chronological sequence by which the energetic parameters of the cell manifest themselves are first a decrease in PCr:Cr followed by an increase in ADP:ATP and an increase in AMP:ATP ratios. However, the mechanisms by which cells may respond to low PCr:Cr and ATP:AMP ratios have only been appreciated after it became obvious that AMP-dependent protein kinase (AMPK) could be activated not only by an increase in AMP:ATP ratio (Winder and Hardie 1999), but also by a lower PCr:Cr ratio (Ponticos, Lu et al. 1998). In terms of the changes in high-energy phosphate levels upon a crisis, the decrease in the PCr:Cr or PCr:ATP ratio represents the first signal seen by cells that are being energetically challenged. Thus the high-energy phosphate-utilizing enzymes, CK and AK, are intricately linked to the AMPK system, in that the substrates or products of these two enzymes can activate the latter protein kinase.

4. The AMP-dependent protein kinase

We have seen in the previous chapter that the first line of defense of the cell to an energy crisis is the phosphocreatine reserves. Creatine kinase is responsible for maintaining the phosphocreatine levels in muscle, which act as an acute energy reserve. Ponticos *et al*. reported that AMPK phosphorylates and inhibits creatine kinase *in vitro* and that AMPK and creatine kinase coimmunoprecipitated (Ponticos, Lu et al. 1998). AMPK is also sensitive to the creatine:creatine-phosphate ratio, such that increased creatine levels stimulate AMPK activity. Conceptually, the regulation of creatine kinase by AMPK is attractive, because it might shut off synthesis of creatine phosphate when ATP levels are compromised and AMP levels increase. Since not all cells need a burst of energy for their function like fast-twitch glycolytic skeletal muscle cells, it makes sense that the signal would be relayed to other energy sensing mechanisms, such as AMPK. AMPK was first discovered as a protein kinase activity associated with HMG-CoA reductase, which was later found to be activated by AMP (Ferrer, Caelles et al. 1985). Parallel studies by Kim and co-workers led to the identification an acetyl-CoA-carboxylase kinase with related properties (Hardie, Carling et al. 1998), but it was Hardie and co-workers who showed that the acetyl-CoA-carboxylase kinase and HMG-CoA-reductase kinase were one in the same enzyme (Carling, Zammit et al. 1987). This, along with the observation that AMPK phosphorylates glycogen synthetase and hormone-sensitive lipase, firmly established it as a multisubstrate kinase. Because AMPK inhibits enzymes involved in glycogen, fatty acid and cholesterol synthesis, it was considered to be primarily a cellular fuel gauge that recognizes ATP depletion and limits further ATP utilization by anabolic pathways (Hardie and Carling 1997). However, it became apparent later that AMPK not only inhibits anabolic pathways, but also initiates a series of compensatory changes that maintain cellular ATP levels. AMPK is activated following ATP depletion or, more accurately, following a rise in the AMP:ATP ratio within the cell. It is important at this point to consider why AMP rather than ADP, should be the key regulatory molecule in monitoring energy status.

AMP:ATP ratios are usually reported, because AMP is a much more sensitive indicator of energy status. Indeed, an important goal for any healthy eukaryotic cell is to maintain low ratios of AMP:ATP or ADP:ATP in the order of 1:100 and 1:10 respectively. These low ratios allow the cell to perform efficient hydrolysis of the anhydride bonds in

ATP which is crucial for the energy-requiring processes of the cell. On the other hand, cells have a very active AK which catalyses the reaction:

2ADP ←→ ATP + AMP

in either direction, such that at equilibrium, AMP:ATP ratios will vary as the square of the ADP:ATP ratios (Hardie and Hawley 2001). In healthy circumstances, cellular ATP levels are abundant and AK will drive the reaction towards the production of ADP, maintaining low levels of AMP. However, in energy crisis ATP levels drop and AK will now drive the reaction towards the production of AMP. Since the AMP:ATP ratio varies as the square of the ADP:ATP ratio, a 5-fold rise in the ADP:ATP ratio, for example, would lead to 25-fold raise in AMP:ATP ratio. This relationship is what allows AMP (or the AMP:ATP ratio) to act as a more sensitive measurement of the cell's energy status, since its concentration would change more dramatically than those of ATP and ADP.

We have seen earlier that AMPK is activated upon an increase in the AMP:ATP ratio and that once activated, its overall effect is to switch on ATP-generating pathways such as fatty acid oxidation and glycolysis (Hardie and Carling 1997) (Hardie, Scott et al. 2003) (Kemp, Mitchelhill et al. 1999). In addition to the acute effects of AMPK on energy metabolism, activation of AMPK has longer-term effects, altering both gene expression (Yang, Hong et al. 2001) and protein expression (Fryer, Foufelle et al. 2002). Although the physiological consequences of these long-term effects of AMPK are not fully understood, it seems likely that they are involved in the overall regulation of energy metabolism. Recent evidence has emerged demonstrating that AMPK is also activated in response to conditions that do not cause a detectable increase in the AMP:ATP ratio, such as treatment of cells with the anti-diabetic drug metformin (Hawley, Gadalla et al. 2002). Nevertheless, this still remains a controversial issue since the absence of changes in AMP:ATP ratio may simply reflect the limitation of sensitive measurements (Hardie 2006) (Hoek 2006). Much of the work on AMPK focused on its regulation of energy levels within individual cells. However, recent findings, suggest that AMPK might have a wider role in regulating whole-body energy metabolism. AMPK is activated in skeletal muscle in response to contraction, resulting in increased glucose uptake (Mu, Brozinick et al. 2001) and fatty acid oxidation (Merrill, Kurth et al. 1997). Two adipocyte-derived hormones – leptin and adiponectin, which themselves play key roles in regulating energy homeostasis – activate AMPK. Leptin activates AMPK in skeletal

muscle, thereby increasing fatty acid oxidation (Minokoshi, Kim et al. 2002), whereas adiponectin activates AMPK in liver and muscle to stimulate glucose usage and fatty acid oxidation, and inhibiting glucose production in liver (Yamauchi, Kamon et al. 2002). Taken together these findings indicate that AMPK might be important in the development of metabolic diseases, such as type 2 diabetes.

AMPK is a heterotrimeric complex comprising a catalytic subunit (α) and two regulatory subunits (β and γ). Homologues of all three subunits have been identified in every eukaryotic species examined to date. This conservation suggests that formation of the heterotrimeric complex is an essential requirement for at least some of the functions of the kinase. In terms of understanding the regulation of AMPK and its function, one of the most significant findings was that AMPK is structurally and functionally related to a protein kinase complex in Saccharomyces cerevisiae termed SNF1 (Hardie, Carling et al. 1998). SNF1 subunits were identified by complementation of yeast mutants that are unable to grow on non-glucose carbon sources, such as sucrose or raffinose (Carlson 1999). Further studies revealed that SNF1 kinase activity is required for the transcriptional activation of many genes that are repressed in the presence of glucose (Carlson 1999). In mammals, isoforms of all three subunits, which are encoded by separate genes, have been identified. The roles of the different subunits within the AMPK complex provide important clues regarding the physiological functions of the kinase, as well as offering valuable insights into its regulation. The N-terminal half of the α subunit contains a typical serine/threonine protein kinase catalytic domain, containing features conserved throughout the protein kinase superfamily (Hanks, Quinn et al. 1988). Expression studies in mammalian cells have indicated that the C-terminal half of the α subunit contains a region of 150 amino acid residues at the extreme C terminus that is required for association with the β and γ subunits, whereas a region immediately downstream of the catalytic domain (residues 312–392 in the α1 isoform) appears to have an inhibitory function (Crute, Seefeld et al. 1998). The α subunit also contains several residues that can be phosphorylated both in vitro and in vivo. One of these residues is the critical residue Thr172 whose phosphorylation is essential for AMPK activity. A few kinases have been found to act upstream of AMPK, as AMPK kinases, such as LKB1, which is discussed in the next chapter and Ca^{2+}/calmodulin-dependent protein kinase. Unlike LKB1, the latter is implicated in the activation of AMPK independently of the AMP:ATP ratio (Hawley, Pan et al. 2005).

5. LKB1 and the Peutz-Jeghers syndrome

As we have seen above, AMPK is the downstream component of a protein-kinase cascade that has an important role in regulating energy homeostasis. Activation of AMPK requires the phosphorylation of Thr172 within the T-loop region of the catalytic subunit α. Until recently, the identity of the upstream T172 phosphorylating kinase was unknown. Several groups independently identified that LKB1 could phosphorylate and activate AMPK (Hawley, Boudeau et al. 2003) (Shaw, Kosmatka et al. 2004) (Woods, Johnstone et al. 2003). Moreover, subsequent studies showed that Ca^{2+}/calmodulin-dependent protein kinases (CaMKKs) can also activate AMPK, suggesting that alternative signaling pathways can also activate this kinase cascade (Hawley, Pan et al. 2005) (Woods, Dickerson et al. 2005). The discovery that LKB1 is an AMPK kinase was unexpected. Previous studies had shown that mutations within the gene encoding LKB1 lead to a rare, dominantly inherited cancer-predisposition syndrome in humans known as Peutz–Jeghers syndrome (Hemminki, Markie et al. 1998) (Jenne, Reimann et al. 1998). This disease is characterized by the development of multiple benign intestinal hamartomas and distinctive pigmentation of the skin and mucous membranes. Peutz–Jeghers patients are at greater risk of developing malignant tumours, particularly of the gastrointestinal tract. However, the role of LKB1 in the regulation of energy metabolism and the role of AMPK in the development of tumour progression were not obvious. Until the discovery that AMPK is a substrate for LKB1, no other physiologically relevant downstream targets for LKB1 were identified. However, analysis of the kinase activity of LKB1 showed that this kinase phosphorylates and activates 12 other kinases in addition to AMPK (Lizcano, Goransson et al. 2004). These kinases, termed AMPK-related kinases, share significant sequence similarity with the kinase domain of AMPK. Currently, the physiological role of most of the AMPK-related kinases is poorly understood; however, the finding that LKB1 activates multiple kinases suggests that it might have a role in regulating diverse signaling pathways. Total knock-out of LKB1 is embryonic lethal (Ylikorkala, Rossi et al. 2001) and therefore led Shaw and colleagues to generate a conditional LKB1 knock-out in the liver to study its specific role in gluconeogenesis. Loss of LKB1 substantially reduced AMPK phosphorylation and activity in the liver, indicating that, in this tissue, LKB1 is the predominant upstream kinase in the AMPK cascade. Deletion of LKB1 led to a marked increase in fasting blood glucose levels and impaired glucose tolerance. However, mice lacking LKB1 in the liver

showed a normal response to insulin, indicating that peripheral glucose uptake was not affected (Shaw, Lamia et al. 2005). Investigation of the expression levels of several genes that are involved in hepatic gluconeogenesis in these animals showed that their levels were significantly higher than in control animals. Moreover, the level of expression of the gene encoding peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) was also increased. Pgc1α acts upstream of many target genes, including those involved in gluconeogenesis (Yoon, Puigserver et al. 2001), and is itself under transcriptional control by the cAMP response-element-binding (CREB) protein (Herzig, Long et al. 2001). Moreover, recently CREB has been shown to be under the regulation of the co-activator transducer of regulated CREB activity 2 (TORC2) (Screaton, Conkright et al. 2004).

6. Metformin and diabetes

Metformin (N',N'-dimethylbiguanide) is the most commonly prescribed oral medication for treatment of type II *diabetes mellitus* (T2DM). Plant-derived biguanide alkaloids from *Galega officinalis* were used as an early treatment for metabolic disturbances, but pharmacological application of metformin for T2DM started only in the late 1950s in Europe and in the mid-1990s in North America (Klepser and Kelly 1997) (Krentz and Bailey 2005). Despite decades of extensive use and study, there are still uncertainties regarding the mechanism of action of biguanides. Metformin is generally considered to have an insulin sensitising effect on peripheral tissues, with little or no effect on insulin secretion per se. Insulin target tissues exhibit diminished gluconeogenesis and enhanced glucose uptake and utilisation in treated patients; this improves glucose tolerance and reduces hyperglycemic markers, diminishing the risk of diabetic complications (Klepser and Kelly 1997) (Krentz and Bailey 2005). Two key observations regarding the potential mechanism of action of metformin have been described recently. First, metformin partially inhibits respiratory complex I (NADH:ubiquinone oxidoreductase) activity in the liver and muscle (El-Mir, Nogueira et al. 2000) (Owen, Doran et al. 2000) (Brunmair, Staniek et al. 2004). This property appears to be attributable to all biguanides. Indeed, inhibition of the electron-transport chain by phenformin (N'N'-phenylethylbiguanide) was demonstrated over four decades ago (Steiner and Williams 1958) (Davidoff 1971). Second, in hepatocytes and myocytes,

which represent model cell types for studying insulin action, metformin induces the activation of AMPK (Zhou, Myers et al. 2001) (Musi, Hirshman et al. 2002). However, the molecular mechanisms by which metformin induces AMPK activation are poorly understood. Nevertheless, through activation of AMPK, metformin treatment decreases the expression of gluconeogenic genes and increases fatty acid oxidation in hepatocytes (Zhou, Myers et al. 2001). Recently, Shaw et al. demonstrated that metformin cannot lower blood glucose level in mice that lack hepatic expression of LKB1. The simplest interpretation of this finding is that LKB1 is required for activation of AMPK by metformin, and that, in turn, AMPK activation is required for decreasing the expression of gluconeogenic genes, via phosphorylation of TORC2, and decreasing glucose output. The role of LKB1 in regulating energy metabolism is well established; however, whether this is entirely due to its ability to activate AMPK is still not clear. As stated earlier, LKB1 activates 12 AMPK-related kinases; determination of their role in regulating metabolic pathways in energy metabolism and cell proliferation will be important in futures studies.

7. mTOR pathway components and functions

The early days of the study of rapamycin, an antibiotic effective against fungi, showed that this compound had potent anti-proliferative and immunosupressive properties in mammalian cells. The target of rapamycin (TOR) was originally discovered in the budding yeast through mutants that showed growth resistance to its effect, leading to the isolation of two functionally distinct protein complexes containing different TOR protein, TOR1 and TOR2 (Heitman, Movva et al. 1991; Helliwell, Wagner et al. 1994). Indeed, in yeast either TOR1 or TOR2 can form TOR Complex1s (TORC1), which also contain KOG1, LST8, and TCO89 and are characterized by their sensitivity to rapamycin. TOR2, however, also forms an additional complex, TOR Complex2 (TORC2), which contains AVO1, AVO2, AVO3, LST8, and BIT61 and is characterized by its resistance to rapamycin (Loewith, Jacinto et al. 2002). Unlike yeast, all other eukaryotes have only one TOR gene, and thus the two TORCs are formed only with one TOR protein that is the functional homolog of yeast TOR2. Although the two TORCs in yeast have distinct functions, they are both associated with the regulation of cell cycle. TORC1 regulates protein synthesis and couples cell size to cell-cycle progression, whereas TORC2 regulates cell-cycle-dependent polarization of the actin cytoskeleton.

KOG1 and AVO3 are the major determinants of TORC1- and TORC2-specific activities, respectively, and both proteins are essential for yeast cell viability. However, the fact that two yeast proteins, TOR1 and TOR2, are dedicated to form TORC1, whereas only one protein, TOR2, can form TORC2, may suggest the relative importance of the two complexes for cell metabolism and viability.

TORC1 and TORC2 are structurally and functionally conserved in mammals (Wullschleger, Loewith et al. 2006). Mammalian TOR (mTOR) also exists in two functionally distinct complexes: mTOR Complex1 (mTORC1) and mTOR Complex2 (mTORC2). mTORC1 is rapamycin sensitive, forming an intracellular inhibitory complex with the peptidyl-prolyl cis–trans isomerase FKBP12 (FK506-binding protein). This inhibitory drug/receptor complex binds to the FKBP12-rapamycin (FRB) domain located N-terminal to the kinase domain of mTOR (Fingar and Blenis 2004). Transcriptional profiling of rapamycin treatment of yeast, Drosophila and mammalian cells shows that the drug affects expression of approximately 5% of all genes in the genome, indicating that TOR has a broad impact on cellular function (Guertin, Guntur et al. 2006). mTOR Complex1 contains mTOR, raptor (the KOG1 equivalent in yeast), mLST8 (also known as GbL, G protein β-subunit-like protein) and PRAS40 (proline-rich AKT substrate 40 kDa) (Hara, Maruki et al. 2002) (Kim, Sarbassov et al. 2002) (Loewith, Jacinto et al. 2002) (Kim, Sarbassov et al. 2003) (Sancak, Thoreen et al. 2007) (Vander Haar, Lee et al. 2007) (see Figure 4). Like TORC1 in yeast, mTOR Complex1 is a major regulator of ribosomal biogenesis and protein synthesis (Hay and Sonenberg 2004). mTOR Complex1 regulates these processes largely by the phosphorylation and inactivation of the repressors of mRNA translation 4E-binding proteins (4E-BPs) and by the phosphorylation and activation of ribosomal S6 kinase (S6K1). The phosphorylation status of 4E-BP1 and S6K1 are commonly used to evaluate mTOR Complex1 activity in vivo. mTOR is a 290 kDa Ser/Thr kinase of the phosphatidylinositol 3-kinase related protein kinase (PIKK) family. Raptor positively regulates mTOR activity and functions as a scaffold for recruiting mTOR Complex1 substrates (Kim, Sarbassov et al. 2002). PRAS40 negatively regulates mTOR Complex1 activity in a manner that depends upon its phosphorylation state (Sancak, Thoreen et al. 2007; Vander Haar, Lee et al. 2007). In contrast to raptor and PRAS40, the molecular function of mLST8 is still unclear (Guertin, Stevens et al. 2006).

In contrast to mTOR Complex1, mTOR Complex2 contains mTOR, mLST8, rictor (AVO3 in S. cerevisiae), mSin1 and the recently isolated PROTOR1 and PRR5, and is

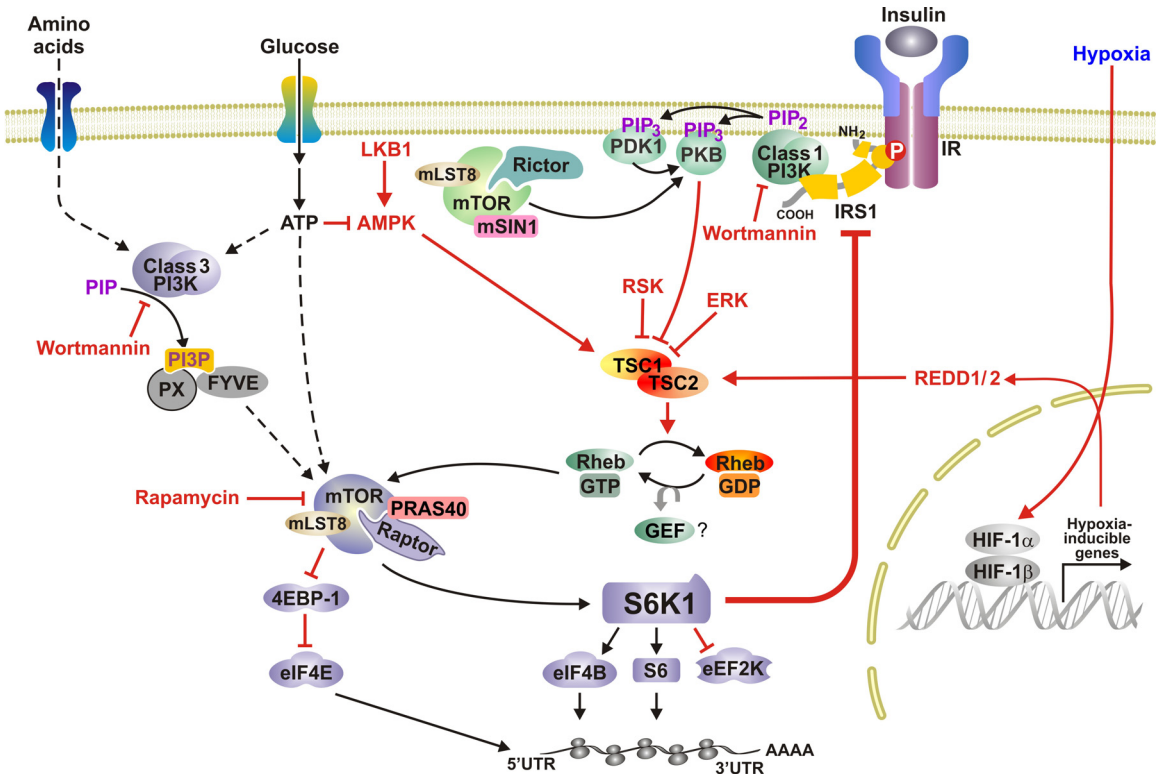


Figure 4: Model of mTOR/S6K1 signaling pathway.

mTOR exists in two structurally distinct complexes: mTOR Complex1 which contains raptor, mLST8, PRAS40, is regulated by inputs including insulin, amino acids, ATP; and mTOR Complex2 which contains rictor, mLST8, mSIN1, which is responsible for transducing the insulin response to PKB by phosphorylating the S473 residue.

Abbreviations: eEF2K, eukaryotic elongation factor 2K; eIF4B, eukaryotic initiation factor 4B; FYVE, Fab1/YOTB/2K632.12/Vac1/EEA1 domain; GEF, guanosine nucleotide exchange factor; PX, Phox homology domain

believed to be rapamycin insensitive (Sarbassov, Ali et al. 2004) (Frias, Thoreen et al. 2006) (Pearce, Huang et al. 2007) (Woo, Kim et al. 2007) (see Figure 4). However, recent results show that prolonged (over 24 hours) rapamycin treatment can disrupt mTOR Complex2 assembly and function by sequestering newly synthesized mTOR molecules (Sarbassov, Ali et al. 2006). mTOR Complex2 mediates PKB/Akt S473 phosphorylation, one of two phosphorylated residues required for full PKB/Akt activation (Alessi, Andjelkovic et al. 1996) (Sarbassov, Guertin et al. 2005).

8. Regulation of the mTOR Complex1 signaling by hormones and nutrients

Growth factors and hormones, such as insulin, initiate mTOR Complex1 signaling by the sequential activation of class 1 PI3K and PKB/Akt, with the latter acting to reverse the inhibitory effects of Tuberous Sclerosis Complex 1/2 (TSC1/2) and PRAS40 on mTOR Complex1 signaling (Vander Haar, Lee et al. 2007). TSC1/2 normally functions to suppress the activation of Ras homolog enriched in brain (Rheb), which is required for mTOR Complex1 activity (Buerger, DeVries et al. 2006) (Long, Ortiz-Vega et al. 2005), whereas suppression of PRAS40 relieves its direct inhibitory effects on mTOR Complex1 (Vander Haar, Lee et al. 2007) (Um, D'Alessio et al. 2006). It has been known for several years that culturing cells under low nutrient conditions leads to a strong reduction in S6K and 4EBP1 phosphorylation, a result that is consistent with the role of mTOR Complex1 in translation regulation (Hara, Yonezawa et al. 1998). The Nutrient sensing function of TOR has been shown to be highly conserved in all eukaryotic organisms tested, including plants, yeast and the fly. However, despite the strong conservation of TOR as a nutrient sensor, there is a significant lack of agreement on a unifying mechanism to explain how TOR is regulated in response to nutrients. Indeed, it has been proposed that nutrients activate mTOR in both a TSC1/2-dependent (Gao, Zhang et al. 2002) and TSC1/2-independent (Smith, Finn et al. 2005) (Nobukuni, Joaquin et al. 2005) fashion. Some studies suggest that Rheb is integral to nutrient sensing, as overexpressed Rheb has been shown to override nutrient deprivation signals to mTOR (Inoki, Li et al. 2003) (Garami, Zwartkruis et al. 2003), and nutrient deprivation signals may decrease Rheb binding to mTOR (Long, Ortiz-Vega et al. 2005).

Other studies imply that Rheb may not be involved at all, but rather that nutrient poor conditions lead to increased mTOR/raptor binding and subsequently decreased mTOR Complex1 activity (Kim, Sarbassov et al. 2002). Recent studies from our laboratory showed that unlike insulin, amino acids and glucose, mediate mTOR Complex1 signaling through class 3 PI3K, or hVps34 (Byfield, Murray et al. 2005) (Nobukuni, Joaquin et al. 2005), rather than through the known components of the generic class 1 PI3K pathway. Depleting hVps34 protein levels via siRNA treatment ablates mTOR Complex1 signaling induced by amino acids (Nobukuni, Joaquin et al. 2005) (Byfield, Murray et al. 2005) or glucose (Byfield, Murray et al. 2005). Moreover, hVps34 was shown to be activated by the presence of amino acids and glucose although the mechanism by which it regulates mTOR Complex1 has yet to be elucidated (Nobukuni, Joaquin et al. 2005) (Byfield, Murray et al. 2005).

9. Regulation of the mTOR Complex1 signaling by cellular energy

Translation and ribosome biogenesis along with the maintenance of osmotic neutrality, are among the most energetically demanding processes that a cell must perform (Schmidt 1999). Therefore, it is not unexpected that these processes must be closely regulated during times of low ATP or nutrient availability. Studies on the role of acute (minutes) and chronic (hours) energy depletion on mTOR Complex1 signaling have largely relied on the use pharmacological agents. Initial studies from our laboratory demonstrated an acute inhibition of mTOR Complex1 signaling in response to the inhibition of glycolytic flux and ATP production (Dennis, Jaeschke et al. 2001). Moreover, in vitro mTOR Complex1 possessed a high Km for ATP in the presence of Mg^{2+}, hence it was hypothesized that real-time, direct sensing of ATP by mTOR Complex1 could be one mechanism whereby intracellular levels of ATP were homeostatically maintained in a narrow range. However, later studies favored a model whereby inhibition of glycolytic flux leads to increased levels of intracellular ADP, which is used by AK to produce ATP and AMP, the latter triggering the activation of AMPK (Inoki, Zhu et al. 2003; Corradetti, Inoki et al. 2004; Shaw, Bardeesy et al. 2004). AMPK acts to further stimulate catabolic pathways that generate ATP, while down regulating major anabolic processes, such as

protein synthesis and ribosome biogenesis, which consume ATP (Carling 2004). In the case of inhibition of mTOR Complex1 signaling, AMPK phosphorylates TSC2 at two specific residues (Inoki, Zhu et al. 2003), which are hypothesized to increase its GAP activity, driving Rheb into the inactive GDP-bound state (Inoki, Zhu et al. 2003; Corradetti, Inoki et al. 2004; Shaw, Bardeesy et al. 2004). The chronic response to energy depletion is controled by Regulated in Development and DNA damage response gene 1 (REDD1), but its dependency on AMPK is not clear (Sofer, Lei et al. 2005). Although absence of REDD1 does not alter AMPK activation, or its ability to phosphorylate TSC2, it ablates the chronic inhibitory effects of energy depletion on mTOR Complex1 signaling. During chronic energy depletion REDD1 is transcriptionally up-regulated which attenuates mTOR Complex1 activity in a TSC1/2 dependent manner. Neither the mechanism of REDD1 transcriptional induction nor the mechanism of action on mTOR Complex1 signaling is known.

Given the key role of mTOR Complex1 in cell growth and metabolism, it is reasonable to predict an association between mTOR Complex1 activity and aberrant pathological states, including diabetes and cancer. The importance of elucidating the fundamental responses of mTOR Complex1 to energy depletion in such disease states is underscored by a case-control study from record-linkage databases demonstrating that patients treated with the anti-diabetic metformin, an AMPK activator, which blunts mTOR Complex1 signaling, reduces the risk of cancer in diabetic patients in a dose dependent manner (Evans, Donnelly et al. 2005; Evans, Ogston et al. 2006).

III. MATERIALS AND METHODS

1. Preparation of reagents

2-deoxy-α-D-glucose was prepared directly in DMEM high glucose containing 50 μg/ml of Penicillin and Streptomycin mix at a stock concentration of 2 M and aliquots were kept at -20°C.

10% SDS PAGE gel was prepared by mixing 3.3ml of a 30% acrylamide/0.8% bis-acrylamide solution, 5ml [Tris(hydroxymethyl)aminomethane]-base buffer (Tris-base) 1.5M pH8.8, 1% sodium dodecyl sulphate (SDS), 100 μl ammonium persulfate (APS) 10%, 10 μl N,N,N',N'-tetramethylethylenediamine (TEMED), topped up with water to 10ml and a 4% stacking gel containing 0.6ml of a 30% acrylamide/0.8% bis-acrylamide solution, 2.25ml Tris-base 0.5M pH6.8, 1% SDS, 15 μl APS 10%, 12 μl TEMED, topped up to 4.5ml with water.

AICAR was dissolved in water at a stock concentration of 200 mM and aliquots were kept at -80°C.

Egg lysis buffer (1X) was prepared by mixing 50 mM Tris-base, 0.1% Igepal CA-630 (Nonidet NP-40), 120 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 6 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 20 mM sodium fluoride, 1 mM sodium pyrophosphate, 30 mM 4-nitrophenyl phosphate, 1 mM benzamidine and adjusted to pH 7.5. This buffer was commonly made as a 2X stock solution and kept at 4°C. Just before extraction, 1X working solution was supplemented with one tablet of EDTA-free protease inhibitor cocktail per 25 ml of buffer and kept on ice.

Metformin was prepared directly in DMEM high glucose containing 50 μg/ml of penicillin and streptomycin mix at a stock concentration of 250 mM and aliquots were kept at -80°C.

Oligomycin A, B, C was dissolved in ethanol (average calculated MW: 802.64) at a stock concentration of 2 mM and kept at -20°C in a tight seal microfuge tube.

Phenformin was dissolved in DMSO at a stock concentration of 1M and aliquots were kept at -80°C.

Laemmli sample buffer 5X was prepared by mixing 125 mM Tris-base, 4% SDS, 20% glycerol, 0.04% bromophenol blue and kept at RT. Just prior use, 10% β-mercaptoethanol was added to the mix.

Transfer buffers for SDS PAGE gels were part of a 3 system buffers which were "anode I' buffer: 300 mM Tris-base, 20% methanol; 'anode II" buffer: 25 mM Tris-base, 20% methanol and "cathode" buffer: 25 mM Tris-base, 40 mM 6-aminocaproic acid, 20% methanol.

2. Cell culture and treatments

All cell lines described were grown in DMEM high glucose supplemented with 10% fetal bovine serum and 50 μg/ml of a Penicillin and Streptomycin mix or as otherwise stated. Cells were maintained in 10cm Petri dishes and split every other day at 1/40 dilution and incubated at 37°C in a wet atmosphere enriched with 5% CO_2. Cells were harvested from the plate using a 0.25% trypsin solution by first washing them with this solution and then incubating for 5min at 37°C. The detachment was further helped by gently pipeting the cells up and down few times onto the dish, to finally harvest them in pre-warmed medium as described earlier. An aliquot of these cells were then counted using a hemacytometer chamber and split to the desired dilution.

Most of the treatments were performed in a 6 wells plate format except for the long time course studies where 6cm Petri dishes were used. In all cases, 100000 cells were seeded 48h before treatment and the medium volume was equally adjusted all across the wells or the plates, by discarding the excessive amount of this original medium. Treatments were performed by adding the reagents directly into the medium and swirling the dish gently several times. Unless otherwise stated, 2-deoxy-α-D-glucose treatments were performed for 30 min at 100 mM, Oligomycin treatments were performed for 30 min at 10 μM, Phenformin at 6 mM for 1h and Metformin at 10 mM for 24h. For insulin stimulation, cells were first starved for serum overnight and then stimulated with 200 nM insulin for 30 min.

For the AMPK overexpression studies, 1μg of each WT α, β and γ subunits of AMPK as well the γ R70Q and R172Q mutants were co-expressed with 0.1ug of GST-S6K reporter

in HEK293 cells in 6cm dishes. All cDNA were transfected with Fugene 6 (Roche) according to manufacturer's instructions.

3. Preparation of protein extracts from cells, gel electrophoresis and Western blotting

Upon completion of the treatments, cell plates were placed on ice and the medium was discarded by vacuum suction. Cells were then washed twice with ice cold PBS and plates were left few minutes on ice in a tilted position to remove any residual buffer. Total protein content was then extracted from cells by typically applying 60 µl of "egg lysis buffer" per well of a 6 wells plate. After an incubation of 5min on ice, cells were then scrapped off the plate and incubated for a further 2min on ice, again in a tilted position to drain extract to the bottom of the well. The cell extract was then harvested and kept at -80°C, typically overnight or until use. The soluble protein fraction was separated from the cell debris with a 10min centrifugation at 14000rpm and 4°C. The protein concentration of the extract was then determined with the bicinchoninic acid (BCA) assay method according to manufacturer's protocol and the extract was diluted to 1 µg/µl with 1X Laemmli sample buffer, boiled for 3min and either kept at -20°C for later use or 30 µg loaded in a SDS PAGE gel. Typically, samples were run in a 10% gel for 1.5h at constant amperage of 60mA. Separately, 4-20% gradient Tris-HCl pre-cast gels purchased from Bio-Rad have also been used throughout this work and were run at a constant voltage of 200V for 1h. In all cases, once the run was completed, the gel slab was soaked in cathode buffer and kept on a rocking platform for 5-10min. During that time, the semi-dry gel transfer was arranged by first lying 3 chromatography papers pre-soaked in anode I buffer onto the anode plate of the transfer apparatus and then stacking on top them in the following order: 3 papers pre-soaked in anode II buffer, PVDF membrane pre-soaked in methanol and rinsed few times in water, the gel slab, 3 papers pre-soaked in cathode buffer and finally on top the cathode plate of the transfer apparatus. The protein transfer was carried out for 1.5 hours at 1.2mA/cm^2 of membrane area and the PVDF filter was then briefly rinsed once with water and once with TBS buffer containing 0.1% (v:v) Tween-20 detergent (TBST). The membrane was then incubated in 5% (w:v) blocker containing TBST, rocking for 30min at RT then briefly

rinsed with TBST and finally incubated with the desired primary antibody rocking at 4°C overnight. Primary antibodies used in this work were: 4E-BP1 antibody; β-actin; ACC phospho-S79; AMPK phospho-T172; AMPK α1,α2; HKII; S6K phospho-T389; S6K; TSC2; tubulin; which all were diluted at 1:1000 (v:v) in TBST containing 5% (w:v) bovine serum albumine and 0.05% sodium azide. Following this incubation, the primary antibody was saved at 4°C until its next use and the membrane was briefly washed with TBST followed by another 3 washes with for 10min at RT. The membrane was then incubated with secondary HRP antibody (1:2500 for anti-rabbit, 1:5000 for anti-mouse and anti-goat) in a solution of TBST containing 0.5% blocker for 1h at RT. Washes were then performed as described earlier for the primary antibody. The membrane was then incubated in ECL solution for 1min and put in between transparent sheet and exposed to photographic film.

4. Drosophila cell culture, RNA interference and DNA sequence analysis.

Drosophila Kc167 cells were maintained as described (Radimerski, Montagne et al. 2002). Protein extracts of cells were prepared and kinase activity of dS6K was measured essentially as previously described (Oldham, Montagne et al. 2000). H2B was used as a substrate for the assay. Treatment with dsRNAi was performed essentially as described (Clemens, Worby et al. 2000), with an incubation time of 7 days. All primers were designed starting with the T7 RNA polymerase binding site as follows: 5'-TTAATACGACTCACTATAGGGAGA-3'. dTsc1; accession no. AF173560, sense-primer 436-453, anti-sense-primer 1081-1098, dTsc2; accession no. AF172995, sense primer 591-608, antisense primer 1371-1388.

Alignments of the TSC2 sequences and AMPK phosphorylation motifs search were carried out using Vector-NTI software (InforMax).

5. Adenine nucleotides measurement by luminometry and HPLC

ATP measurements by luminometry were carried out using the ATP bioluminescence assay kit CLS II from Roche and performed as specified in the manufaturer's protocol
For the measurement of AMP, ADP and ATP, cells were grown typically in 6cm dishes and were treated as above (point 2 of Materials & Methods). The medium was discarded and the cells were washed twice with PBS. Cells were then washed twice with ice cold PBS and plates were left few minutes on ice in a tilted position to remove any residual buffer. Cells were then extracted with 60 µl of perchloric acid 1N and immediately scrapped off the plate and the liquid placed in microfuge tube. The extract was further incubated on ice for another 5 min and was then centrifuged at 4°C for 5 min. The supernatant was isolated carefully and immediately neutralized with a 1:4 (v:v) mix of bromophenol blue and 3M K_2CO_3 by adding a tenth of the volume of the supernatant, or until the sample became green to blueish but avoiding yellow color. Samples were then centrifuged at 14000rpm and RT and were further filtered through for residual precipitate. The filtrate was either analysed straight away or kept at -80°C until use. The chromatographic system used was from Shimadzu instruments Class V-P .20 µl of samples were injected into the anion exchange column Partisphere 5 SAX from Whatman and separated using buffer A: 10mM $(NH_4)H_2PO_4$ pH3.7 and buffer B: 480mM $(NH_4)H_2PO_4$ pH3.7. The separation method was 8 min 0% buffer B, 12 min 5% B, 15 min 35% B, 20 min 45 % B, 25 min 50% B, 27 min 100% B, 43 min 100% B and 44 min 0% B. The flow rate was 1.25 ml/min and the detection was performed with UV Vis detector at 259 nm. Data collection and analyses was carried out using the software EZStart version 7.3.

6. Measurement of mitochondrial membrane potential by flow cytometry

The fetal liver-derived hematopoietic cell line FL5.12 was grown in suspension in T75 flasks with RPMI medium supplemented with 10% FBS, 20 mM HEPES buffer, 50 μg/ml of a Pen/Strep mix, 50 μM L-Glutamine, 50 μM 2-mercaptoethanol and 0.35 ng/ml recombinant interleukine-3, and split every other day when they reach 300000 cells/ml.
For the measurement of the mitochondrial membrane potential ($\Delta\Psi_m$), cells were split into 14 ml conical tubes at a density of 500000 cells/ml and treated as desired (see §2 above). Tetramethylrhodamine ethyl ester (TMRE) potentiometric dye was added at least 15 min prior measurement at 20 pM. 4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA and was used to stain the nucleus of the cell. It was used at 2 μg/ml final but is optional. As a positive control, the organic acid carbonylcyanide – m – chlorophenylhydrazone (CCCP) was used to abolish the $\Delta\Psi_m$ and was used at 50 μM final. The measurement of $\Delta\Psi_m$ was carried out using the Becton Dickinson ARIA instrument and analyses were performed using the BD FACS Diva software.

7. In vitro hVps34 kinase activity assay

The number of plates and treatments were carried as desired (see §2 above). Cells were harvested on ice, washed with cold PBS and extracted with "Cantley Lysis Buffer" (CLB) (1ml CLB for 10cm plate). Cantley Lysis Buffer: 1% NP-40, 150mM NaCl, 50mM Tris pH7.4, 10% Glycerol, 10mM NaF, 1mM NaPyrophosphate, protease/phosphatase Inhibitors. Cells extracts were spun down and cleared from its cell debris. 1ml of cell lysate was used for immunoprecipitation with 1μg of C-terminus α-VPS34 antibody (in-house) which could take place between 2 h to overnight at 4°C. Following this incubation, 60μl of 50% slurry of blocked protein A was added and immune complexes were pull-down for 1h at 4°C. The blocked protein A was prepared as following: rinse out 1 bottle of protein A Sepharose (GE Healthcare- 17-0469-01) let it settle down and pour off the supernatant. Re-suspend the protein A Sepharose in 1% BSA, 10mM Tris pH 7.5 and rotate for 30 min RT, gently spin down beads and wash

three times in milli-Q water. Finally, re-suspend the beads in 1:1 cold PBS, aliquot are stored at 4°C.

Before starting the washes of the immunoprecipitates (IP), a silica-aluminum plate is dried up in the oven at 100°C. IP's are washed 4 times in 1x PBS - 1%NP-40, 4 times in 100 mM Tris pH7.4, 500 mM LiCl and 3 times in TNE (10mM Tris 100 mM NaCl 1 mM EDTA). IP's are finally re-suspended in 60µl TNE. The substrate phosphatidylinositol (PI) was prepared by using 2 µl of Avanti PI in chloroform for each IP plus enough for two extra IP's i.e. 10 IP's = 20µl PI + 4µl extra = 24µl total. Blow off the chloroform using an Argon or Helium stream and re-suspend PI in 120µl of Tris-EGTA (10µl for each sample 10 mM Tris 1 mM EGTA). Sonicate PI in a water bath sonicator for 10min. Leave at RT after sonication.

Prepare 100 mM $MnCl_2$ enough for 10 µl for each IP plus two (i.e 120µl for 10 IPs). Add 3 µl of 1 mM elution peptide (immunizing peptide) to each IP and incubate 30°C for 30 min and mixing every 10 min. The eluate is kept at RT. Add 10 µl $MnCl_2$ and then 10 µl PI to each IP. In the radioactive room combine 1 µl of cold 10mM ATP, 1 µl hot ATP (Perkin Elmer – NEG502A) and 3 µl of water for each IP plus two extra. For 10 samples – 12µl Cold, 12µl Hot and 36µl water. Start the assay by adding 5 µl of ATP mix to each IP and place on vortex/shaker staggered every ten seconds. Allow the assay to proceed for 10 minutes. Stop the assay by adding 20 µl 8M HCL beginning with the first IP to have ATP added and proceeding through each sample staggered every ten seconds. Extract the IP with beads intact by adding 160 µl of chloroform:methanol 1:1. Briefly vortex each IP and then spin for 5 minutes. Spot dried silica, aluminum plates (EMD Chemicals – 5553-7) using a Drummond capillary pipette and 50 µl capillary pipettes (Fisher - K71900-50) and drying with an air stream through a 200 µl pipette tip. Place Thin layer chromatography plate into running chamber with 100 ml running buffer (60ml chloroform, 47ml methanol, 11.3 ml water and 2ml Ammonium Hydroxide). Run plate for approximately 40 minutes until mobile phase is 1 cm from top of plate. Remove plate from chamber and dry with an air stream. Plastic wrap plates and expose to film for at least one hour (keep exposure cassette in -80°C during exposure).

IV. RESULTS

1. mTOR/S6K signaling pathway and cellular energy

The mTOR signaling pathway lies at an intracellular crossroad that integrates signals coming from growth factors and hormones, such as insulin, and signals from nutrients, such as amino acids and glucose. In addition, it has become more recently evident that an important function attributed to mTOR is its ability to respond to the cell's energy demand in order to adapt the cell to its nutrient status (Dann, Selvaraj et al. 2007) (Corradetti and Guan 2006). The relationship between the cell's energy status and the mTOR pathway was first established through pharmacological approaches using ATP-depleting agents such as the glycolytic inhibitor, 2-deoxyglucose (2DG), and the mitochondrial electron transport chain inhibitor, rotenone (Dennis, Jaeschke et al. 2001). Both inhibitors have been shown to affect the mTOR pathway in a manner that reflects ATP levels in the cell (Dennis, Jaeschke et al. 2001). In agreement with this report, we also found that increasing concentrations of 2DG led to a dose-dependent attenuation of mTOR signaling, as assessed by S6K1 T389 phosphorylation (Figure 1A). Moreover, this decrease in mTOR signaling correlated well with the levels of ATP in the cell (Figure 1B). This 2DG-induced effect on S6K1 T389 phosphorylation and on cellular ATP levels was highly reproducible and also seen in other cell lines, such as HeLa cells and the small cell carcinoma cell line, A549 (data not shown). However, the use of oligomycin, an inhibitor of mitochondria and ATP production, unexpectedly did not result in such a correlation when directly compared to 2DG. Indeed, oligomycin induced a small reduction of ATP (Figure 2B) and a slight increase in the AMP:ATP ratio (Figure 2C and see Introduction §4 for AMP:ATP ratio). Yet, oligomycin substantially induced the dephosphorylation of S6K1 T389 (Figure 2A). This phenomenon was also observed in mouse embryonic fibroblasts (MEFs) (Figure 3A, B, C) and HeLa cells (data not shown). Because we consistently observed a marginal reduction of ATP with oligomycin, and because a small change in these levels was sufficient to produce a pronounced effect on S6K1 T389 phosphorylation, we decided to investigate whether the extent of S6K T389 dephosphorylation would be comparable under similar ATP levels obtained by titrating the concentration of 2DG or oligomycin. The result of this experiment is presented in Figure 4 and shows that in cells treated with increasing concentrations of 2DG, S6K1

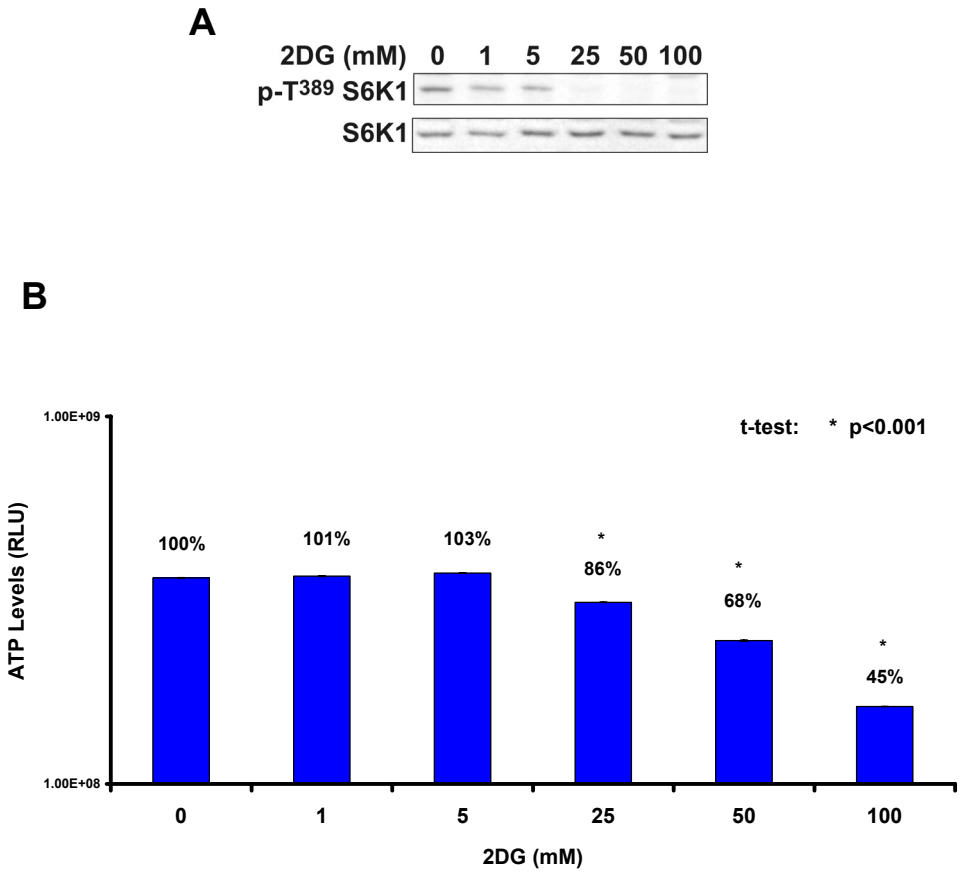


Figure 1: Exponentially growing HEK293 cells were treated with 2DG for 15 min at the indicated concentrations. Parallel plates were extracted either for **A)** Western blotting or **B)** ATP measurements by luminometry.

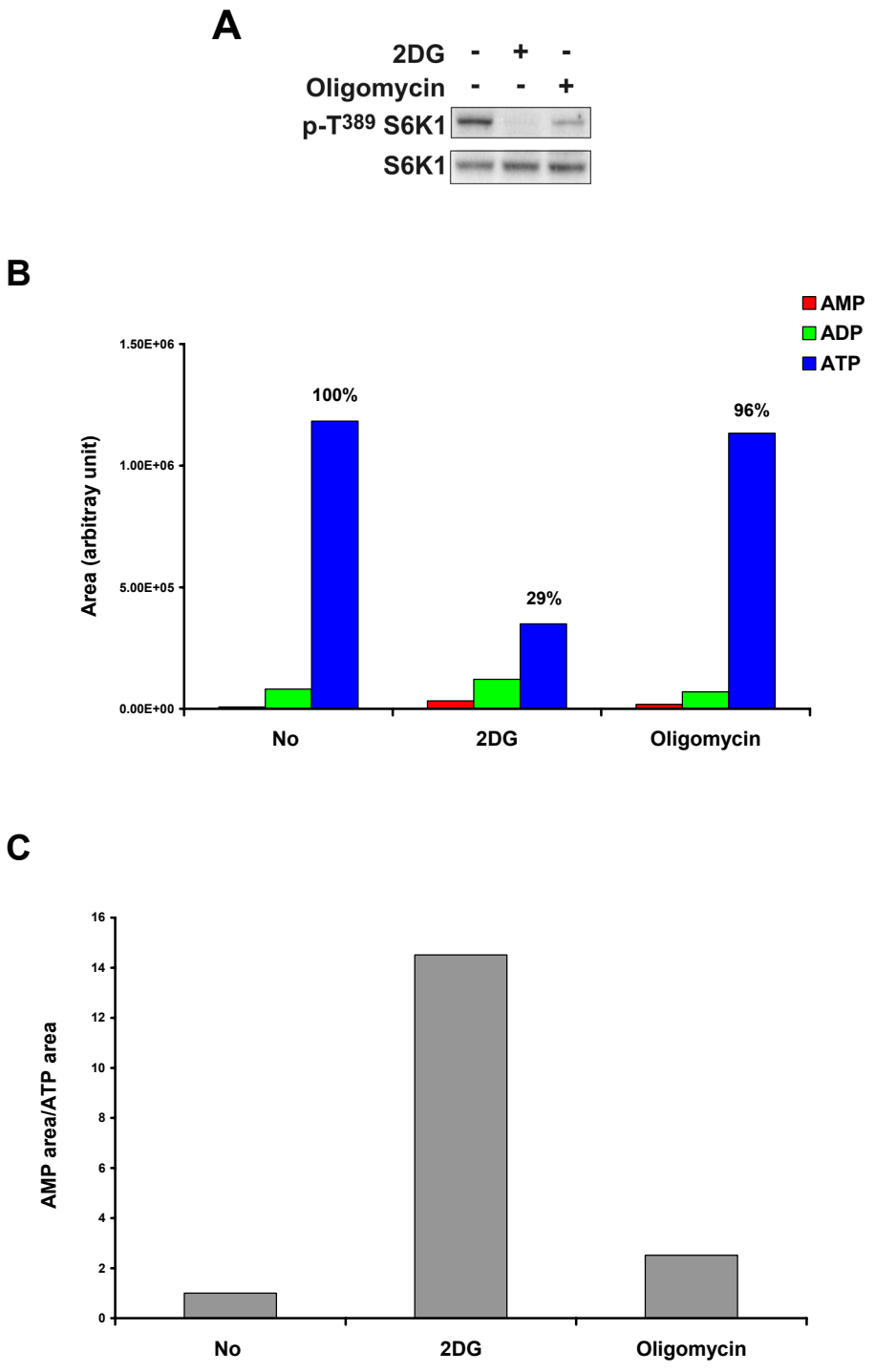


Figure 2: Exponentially growing HEK293 cells treated with 100 mM 2DG or 10 μM oligomycin for 15 min. Parallel plates were extracted either for **A)** western blotting or **B, C)** for adenine nucleotide measurements by HPLC (see Appendix 1 for representative chromatograms of adenine nucleotide measurements by HPLC). Results are representative of two independent experiments.

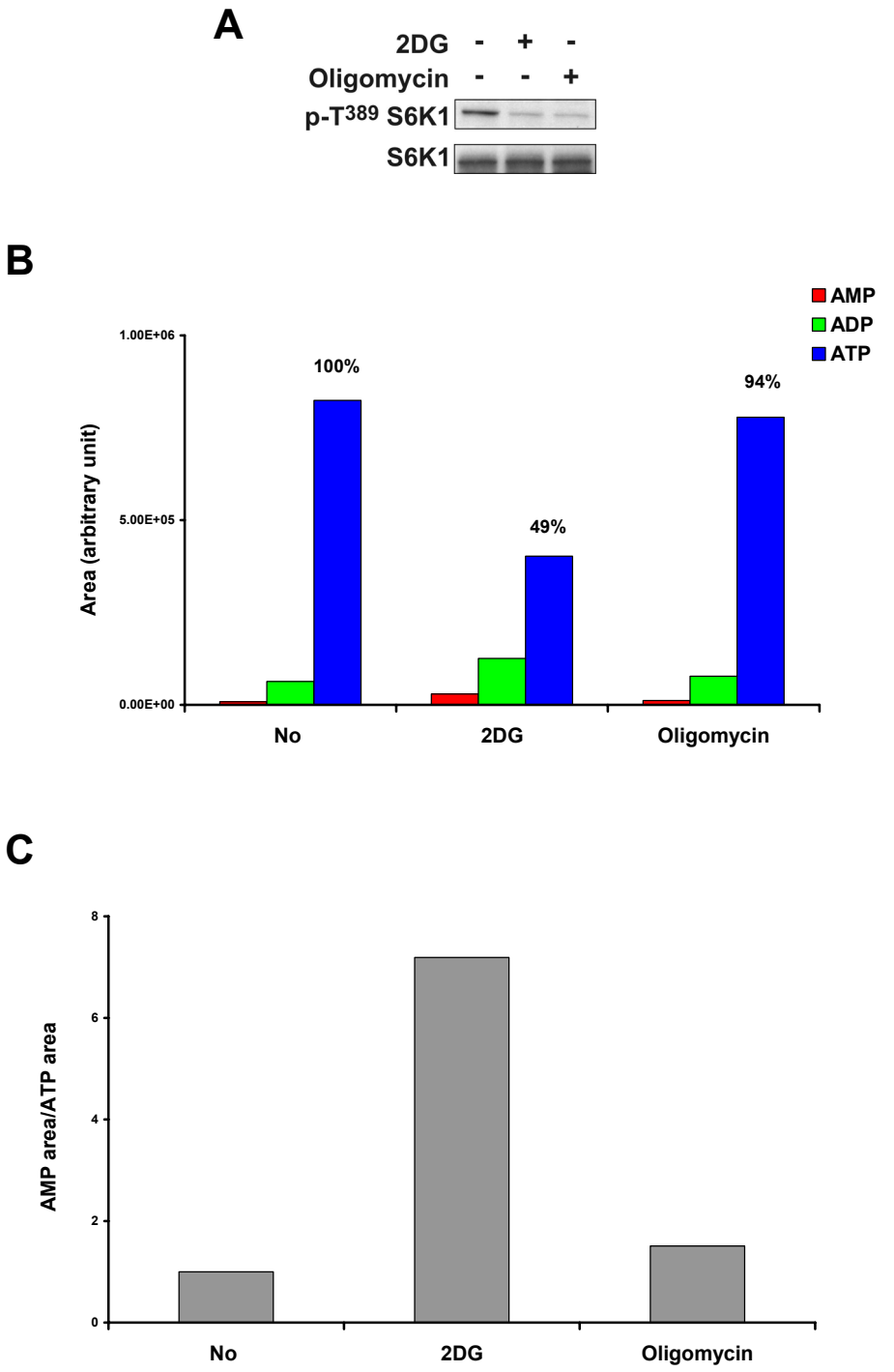


Figure 3: Exponentially growing MEFs treated with 100 mM 2DG or 10 μM oligomycin for 15 min. Parallel plates were extracted either for **A)** Western blotting or for **B,C)** adenine nucleotide measurements by HPLC. Results are representative of two independent experiments.

T389 phosphorylation responded to changing AMP:ATP ratios in a dose-dependent manner. In contrast, oligomycin-treated cells did not show a correlation between AMP:ATP ratio and S6K1 T389 phosphorylation. Moreover, this titration experiment revealed that S6K1 T389 phosphorylation could be decoupled from the AMP:ATP ratios. Indeed, if we look at the AMP:ATP ratio obtained with 10 μM oligomycin treatment, this ratio is roughly equivalent to the AMP:ATP ratio obtained with 12.5 mM 2DG treatment (Figure 4). However, the extent of dephosphorylation of S6K1 T389 showed that oligomycin has a more pronounced effect in blocking S6K1 T389 phosphorylation than 2DG, despite inducing similar increase in the AMP:ATP ratios at these concentrations (Figure 4). This raised the possibility that the effect of oligomycin on the dephosphorylation of S6K1 T389 was independent of the ATP levels as well as on the AMP:ATP ratios in the cell. Similar results were also obtained in MEFs (data not shown). These findings were unexpected as it is well documented that oligomycin interferes with ATP production in the cell by targeting the F_0F_1 ATPase complex or ATP synthetase of the mitochondrial respiratory chain (Boyer 1997; Johnson, Cleary et al. 2006). This raised the possibility that, although we did not see any significant changes in total levels of ATP with oligomycin, the mTOR Complex1 pathway was responding because of its effect on ATP production. To confirm that oligomycin was effectively altering oxidative phosphorylation, and therefore ATP production, we measured the mitochondrion transmembrane potential ($\Delta\Psi_m$) of cells treated with oligomycin and used 2DG as a control. A cytological staining approach was chosen to measure $\Delta\Psi_m$ by using a cationic fluorescent dye, tetramethylrhodamine ethyl ester (TMRE), which diffuses freely across the mitochondrion bilayer membrane to bind the negative charges at the inner membrane that accumulate during electron transport. The amount of dye accumulated in the cell was detected by flow cytometry and was directly proportional to its $\Delta\Psi_m$. Due to the format of this assay, we used a cell line that grows in suspension, the fetal liver-derived hematopoietic cell line FL5.12. Figure 5A shows that treatment of this cell line with 2DG and oligomycin resulted in a dramatic reduction of S6K1 T389 phosphorylation with no apparent difference between the treatments. This result shows that this cell line can be used as a valid model for the measurement of $\Delta\Psi_m$ since it responds to the ATP-depleting agents equally well as those cell lines growing on tissue culture plates. As anticipated, 2DG treatment of these cells reduced $\Delta\Psi_m$ (Figure 5B), due to the dramatic decrease in the flow of metabolites coming from glycolysis and feeding into the TCA cycle. This decrease results in an impaired flow of electrons and accompanying release

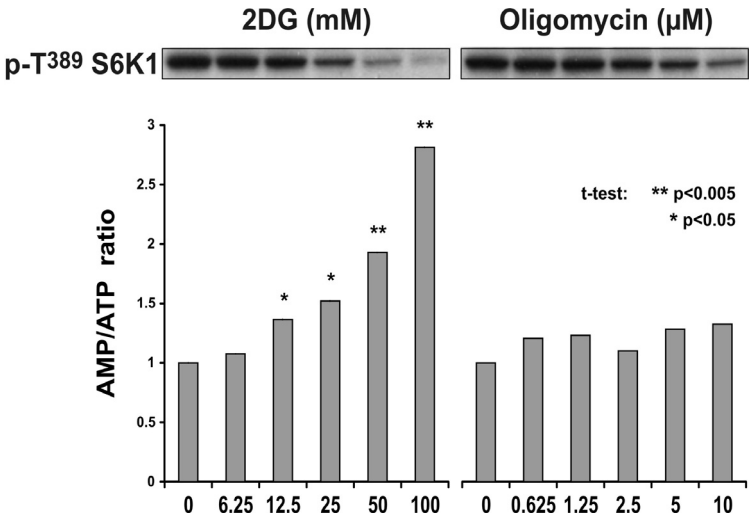


Figure 4: Exponentially growing HEK293 cells treated either with 2DG or oligomycin for 15 min at the indicated concentrations. Parallel plates were either extracted for western blotting or for adenine nucleotide measurements by HPLC.

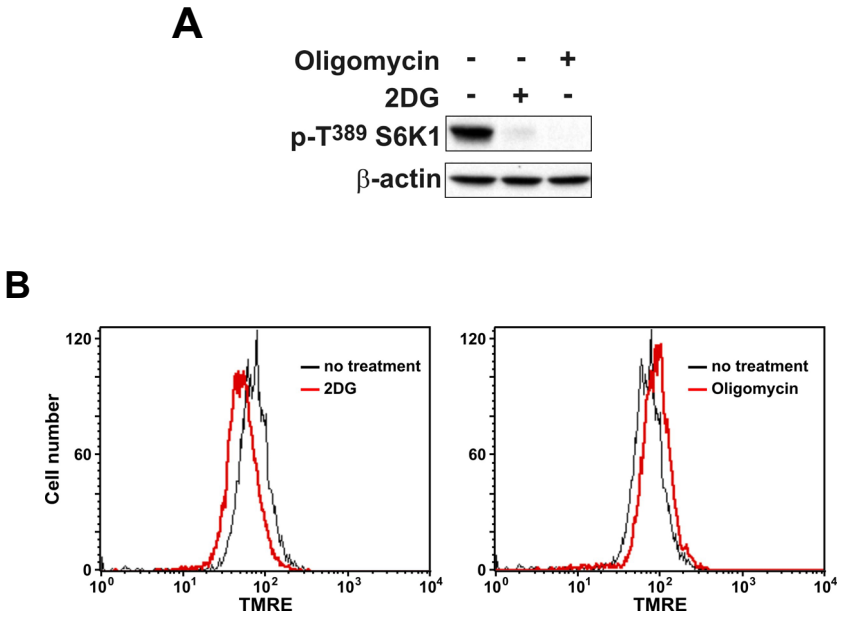


Figure 5: Exponentially growing FL5.12 cells treated with 100 mM 2DG or 10 μM oligomycin for 30 min. For the mitochondrial membrane potential analysis, TMRE was co-treated with the inhibitors for 30 min. Parallel flasks were used either for **A)** extraction and western blotting or **B)** for the measurement of fluorescence emission by flow cytometry.

of protons from the matrix to the intermembrane space, the latter being responsible for maintaining the membrane potential. In contrast to 2DG however, oligomycin treatment had the opposite effect as it increased $\Delta\Psi_m$ in these cells (Figure 5B). This rise in $\Delta\Psi_m$ is explained by the fact that oligomycin blocks the proton channel of the F_0 segment of the ATP synthetase complex and prevents the flux of protons from the mitochondrial intermembrane space to the matrix, which is needed to provide the energy necessary for the F_1 ATP synthetase, thereby maintaining a high membrane potential. Although not intuitive, the finding that 2DG and oligomycin have opposite effects on mitochondrial membrane potential is consistent with the impact that these two inhibitors would have on the production of ATP. Therefore the block of oxidative phosphorylation by oligomycin is anticipated to result in a decrease in ATP levels. Nevertheless, treatments with 2DG and oligomycin showed that only 2DG significantly affected the energy status of the cell, as measured by the total levels of ATP and the AMP:ATP ratios.

2DG and oligomycin have a dominant effect over insulin-induced activation of the mTOR Complex1 pathway as seen by the decrease in S6K1 T389 phosphorylation and by the increase in electrophoretic mobility of 4E-BP1 (Figure 6). Moreover, these inhibitors dramatically abrogated the stimulation of MEFs by amino acids, as demonstrated by the decrease in S6K1 T389 phosphorylation (Figure 7). Taken together the results demonstrate that the ATP-depleting agents affect the mTOR pathway regardless of the growth-promoting stimuli that this pathway transduces, and point out that this regulation might happen at a level proximal to mTOR Complex1.

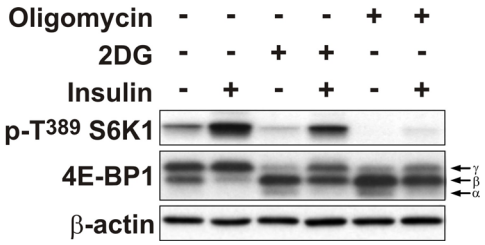


Figure 6: Serum-starved immortalized MEF cells treated with 100 mM 2DG or 10 μM oligomycin for 30 min or co-treated with 200 nM insulin for 30 min.

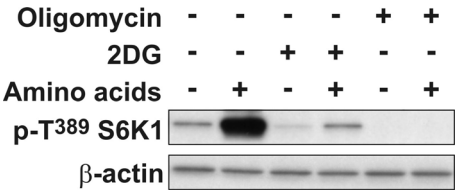


Figure 7: Immortalized MEF cells were serum starved overnight and then amino acid starved for 2h. This was followed by treatment with 100 mM 2DG or 10 μM oligomycin for 30 min or co-treatment with 1X amino acids for 30 min.

To date, all the energy depletion experiments have focused on downstream substrates of the mTOR Complex1 signaling. Initial reports showed that in an in vitro kinase assay, mTOR was responding to increasing concentrations of ATP (Dennis, Jaeschke et al. 2001). However, at the time one could not distinguish whether this in vitro mTOR activity was associated to mTOR Complex1 or mTOR Complex2, since this assay was based on the immunoprecipitation of mTOR itself. Therefore, we wished to check whether mTOR Complex2 was also sensitive to cellular energy levels by checking the phosphorylation of the mTOR Complex2 substrate, PKB/Akt (Sarbassov, Guertin et al. 2005). The result of this experiment showed that 2DG, like rapamycin, was effective in blocking mTOR Complex1 signaling as assessed by the decrease in S6K1 T389 phosphorylation (Figure 8). However, this inhibition was transient as the signal started to recover by 60 min (see below). In contrast to S6K1, treatment with 2DG did not inhibit PKB/Akt S473 phosphorylation but rather increased it (Figure 8). This effect may be attributed to the abrogation of the negative feedback loop mediated by S6K on PI3K signaling (Um, D'Alessio et al. 2006). These findings suggested that unlike mTOR Complex1, mTOR Complex2 is not sensitive to 2DG mediated energy depletion. Together the results obtained above showed that mTOR Complex1 but not mTOR Complex2, is sensitive to ATP levels in the cell and that energy is a critical component of hormone- and nutrient-induced signaling to mTOR.

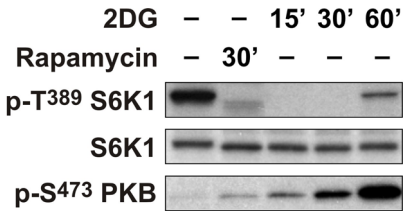


Figure 8: Exponentially growing Hela cells treated either with 100 mM 2DG or 20 nM rapamycin for the times indicated.

2. The TSC1/2 complex is not required for the acute energy signal to mTOR Complex1

mTOR has been shown to be regulated by intracellular ATP levels due to its apparent high Km for ATP as compared to other known serine-threonine kinases. It was postulated that mTOR senses directly the ATP levels in the cell to regulate ribosome biogenesis and cell growth (Dennis, Jaeschke et al. 2001). However, subsequent studies favored a model whereby AMP-dependent protein kinase (AMPK) would serve as a primary sensor for changes in the energy status of the cell. This kinase would be a much more sensitive indicator because its activity is not only sensitive to cellular ATP levels but, particularly, because it can monitor the levels of cellular AMP, the latter varying much more rapidly during energy crisis (see Introduction §4). AMPK plays an important role in the cell's metabolism because an unbalance in the AMP:ATP ratio in the cell, indicative of an energy decline, will trigger AMPK to acutely control metabolic processes and chronically regulate the expression of genes involved in switching the cell from energy-consuming to energy-sparing-processes to restore the cellular ATP levels (Hardie, Hawley et al. 2006) (Carling 2004). Consistent with this model, previous studies have reported that activation of AMPK negatively regulates the mTOR Complex1 signaling pathway in response to energy depletion (Kimura, Tokunaga et al. 2003) (Krause, Bertrand et al. 2002). Further studies showed the existence of a molecular link between AMPK and the mTOR Complex1 whereby AMPK phosphorylates TSC2 at two critical residues, T1227 and S1345, which is thought to enhance the GAP activity of TSC2 towards Rheb, driving it into the GDP-bound state and down regulating the cell growth function of mTOR Complex1 (Inoki, Zhu et al. 2003). Oligomycin has been shown to change the AMP:ATP ratio in *Drosophila* cells and activate dAMPK (Pan and Hardie 2002). However, neither the equivalent AMPK T1227 and S1345 phosphorylation sites nor the domain in which they reside in dTsc2 appear to be conserved in *Drosophila* (Figure 9). This observation suggested that despite the absence of these conserved AMPK phosphorylation sites, there might be additional pathways involved in energy depletion that could potentially operate on the dTOR/dS6K pathway. Moreover, recent work has questioned the role of TSC2 in transducing the acute energy signal to the mTOR Complex1 signaling pathway because 2DG treatment in TSC2-deficient MEFs

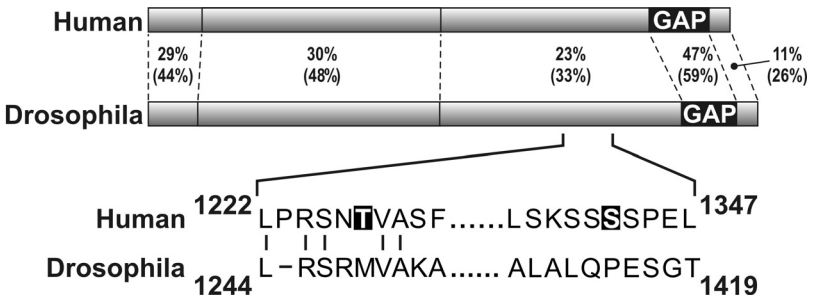


Figure 9: Scheme depicting the sequence alignment of human and *Drosophila* TSC2. Percentages of sequence identity or similarity (in parentheses) of different regions of TSC2 are indicated. The major AMPK phosphorylation sites in TSC2 reported to be affected by energy depletion are shaded. GAP denotes the GTPase-activating domain.

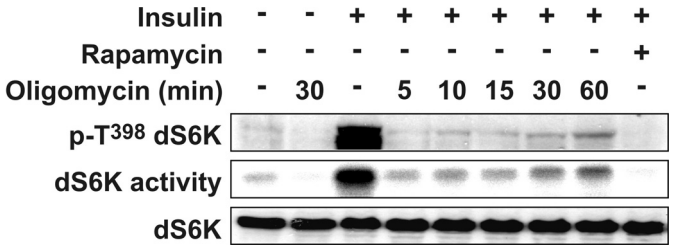


Figure 10: Analysis of dS6K activity following treatment of Kc167 cells with 10 μM oligomycin for indicated time points, 20 nM rapamycin for 15 min, and 100 nM insulin for 30 min. In samples containing insulin plus either 10 μM oligomycin or 20 nM rapamycin, the two inhibitors were added 15 min prior insulin treatment. Western-blot analysis of dS6K T398 phosphorylation and dS6K, top and bottom panels respectively. In vitro dS6K activity is shown in the middle panel.

could affect the mTOR pathway, as detected by a change in the mobility shift of 4E-BP1 (Smith, Finn et al. 2005). Given these reports, and the fact that AMPK phosphorylation sites in dTsc2 are not conserved, we decided to investigate whether dTsc1/2 mediates the effect of acute energy depletion on dTOR signaling. Kc167 cells were treated with oligomycin to increase the AMP:ATP ratio in these *Drosophila* cells (Pan and Hardie 2002) and then dS6K activity and phosphorylation of T398, the *Drosophila* equivalent of the mammalian S6K1 T389, were measured (Figure 10). These results show that, despite absence of the orthologous mammalian AMPK sites in dTsc2, basal levels and insulin-induced activation of dS6K1 are acutely inhibited by oligomycin (Figure 10). Moreover, as seen earlier in Figure 8, dS6K1 T398 dephosphorylation seems to be transient since the signal starts to recover by 30 min of treatment. This indicates that the recovery takes place either because the drug has a short half-life or, because oxidative phosphorylation is blocked and respiration is no longer able to produce ATP, such that the cell switches to anaerobic glycolysis to maintain its ATP levels (see below). Although the AMPK phosphorylation motifs are absent in dTsc2, sequence analysis revealed that there were other potential AMPK phosphorylation sites in dTsc2 (data not shown). This raised the possibility that dAMPK could still phosphorylate other sites in dTsc2 to transduce the acute energy signal to dTOR. To test this possibility, the levels of dTsc1 or dTsc2 were depleted by RNA interference to examine the implication of these proteins in this response. Such treatment has previously been shown to lead to an increase in dS6K activity (Radimerski, Montagne et al. 2002), an effect which was not further augmented by insulin stimulation (Figure 11). However, neither depletion of dTsc1 or dTsc2 had an effect on the ability of oligomycin to inhibit dS6K1 T398 phosphorylation or dS6K1 activity (Figure 11). Depletion of dTsc1 could be readily detected, but depletion of dTsc2 protein could not be examined due to a lack of any available source of antibody recognizing this protein. However, previous reports showed that depletion of one member of the tuberous sclerosis complex leads to the ubiquitination and degradation of the other member of the complex (Benvenuto, Li et al. 2000). Consistent with this report, we observe that dTsc1 levels decrease when we treat cells with dsRNAi directed against dTsc2 (Figure 11, bottom panel). Taken together, these findings indicate that acute energy depletion can suppress *Drosophila* TOR signaling in a dTsc1/dTsc2-independent manner.

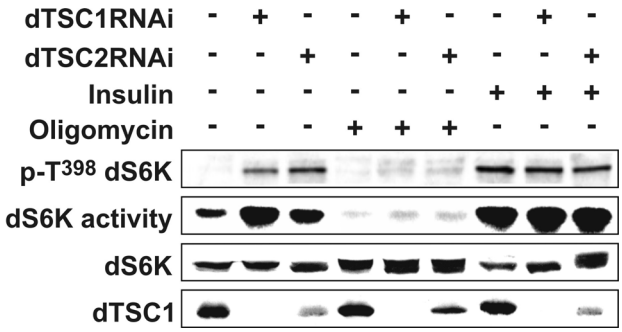


Figure 11: Analysis of dS6K activity following dTSC1, dTSC2 dsRNAi treatment in the absence or presence of either 100 nM insulin or 10 μM oligomycin for 30 min. Western blot analysis of dS6K T398 phosphorylation and in vitro dS6K activity, top panels, respectively. Western blot analyses of dS6K and dTSC1, bottom panels.

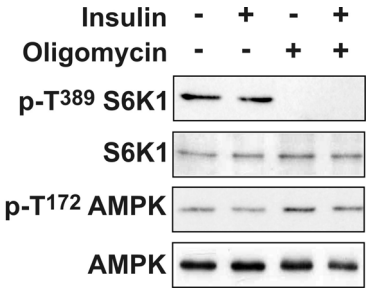


Figure 12: The TSC1-deficient mouse renal carcinoma cell line CACL-1-111 was deprived of serum overnight and stimulated with 200 nM insulin for 30 min. Oligomycin, (10 μM) was added either alone or for 30 min with insulin.

The results obtained above prompted us to question the role of the mammalian TSC complex in mediating the acute energy-depletion signal to the mTOR Complex1 signaling pathway. We, therefore, took advantage of a renal cell carcinoma cell line where expression of TSC1 protein was ablated (Gao, Zhang et al. 2002) (Nobukuni, Joaquin et al. 2005) and examined the ability of oligomycin to inhibit the mTOR Complex1 signaling pathway in these cells. In agreement with previous reports showing that this cell line has constitutively active mTOR Complex1 signaling, insulin stimulation did not augment further S6K1 T389 phosphorylation (Gao, Zhang et al. 2002; Jaeschke, Hartkamp et al. 2002) (Figure 12), consistent with the notion that TSC1/2 act as a tumor suppressor in mediating the insulin signal to mTOR Complex1 (Inoki, Li et al. 2002; Jaeschke, Hartkamp et al. 2002; Manning, Tee et al. 2002; Tee, Fingar et al. 2002). However, addition of oligomycin, either in the absence or presence of insulin, abolished S6K1 T389 phosphorylation, indicating that mTOR Complex1 can be regulated by acute energy depletion even in the absence of TSC1. As reported previously, oligomycin-induced downregulation of S6K1 was accompanied by an increase in AMPK activation, as revealed by increased phosphorylation at the critical residue T172 (Hardie, Scott et al. 2003) (Carling 2004) (Figure 12). In order to check whether the effect of oligomycin on S6K1 T389 phosphorylation accounts for the absence of the TSC1/2 complex as a whole, TSC2-deficient MEFs were tested for the same response (Zhang, Cicchetti et al. 2003). The result of this experiment showed that there was no difference in the energy signaling between the TSC1- and TSC2-deficient cells, and that there was no apparent distinction in the kinetics of S6K1 T389 dephosphorylation between $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs (Figure 13). Moreover, this reduction in S6K1 T389 dephosphorylation was closely paralleled by the increase in AMPK T172 phosphorylation, supporting the role of AMPK as a negative regulator of mTOR Complex1 signaling (Krause, Bertrand et al. 2002; Kimura, Tokunaga et al. 2003). However, the inhibition of the mTOR Complex1 signaling pathway by oligomycin was transient, as S6K1 T389 phosphorylation began to recover by 60 min and AMPK T172 phosphorylation started to decrease at this time (Figure 13). This phenomenon was reported above in Kc167 cells (Figure 10) and was also observed in TSC1-deficient cells (data not shown), supporting the argument that there is no defect in the response of the mTOR Complex1 signaling pathway to the energy deprivation signal induced by oligomycin when the TSC complex is absent.

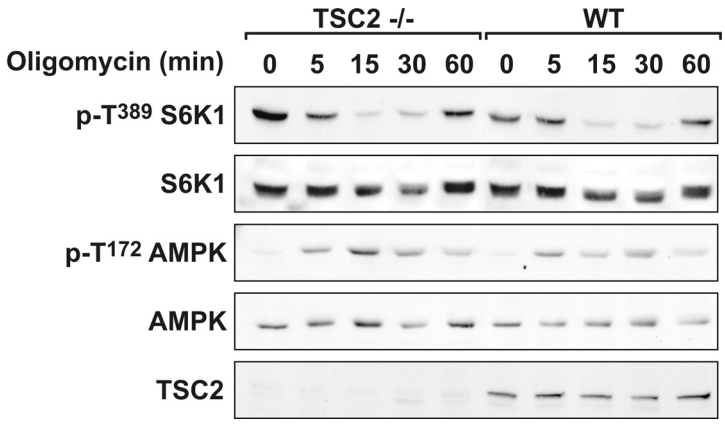


Figure 13: Exponentially growing *TSC2*$^{-/-}$ or *TSC2*$^{+/+}$ MEFs treated with 10 μM oligomycin for the times indicated.

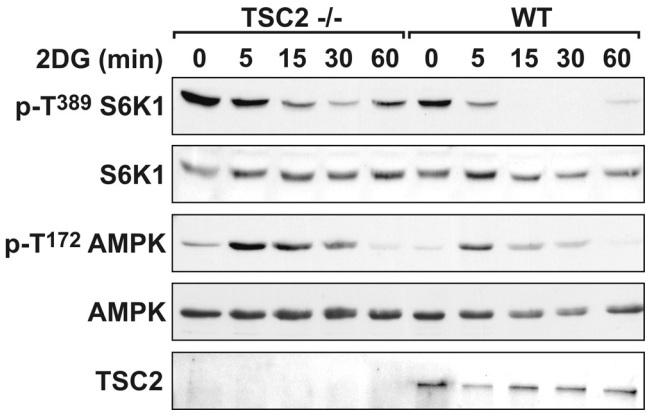
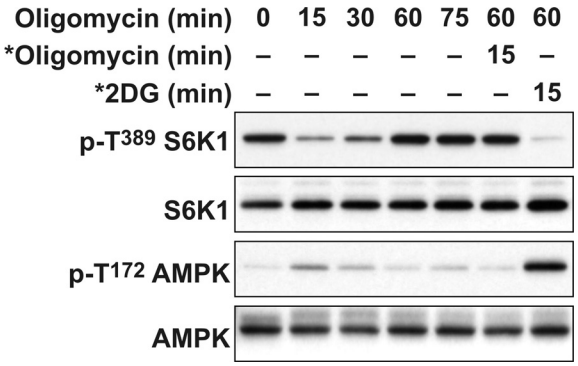


Figure 14: Exponentially growing *TSC2*$^{-/-}$ or *TSC2*$^{+/+}$ MEFs treated with 100 mM of 2DG for the times indicated.

The findings above are quite distinct from those previously reported (Inoki, Zhu et al. 2003), which were primarily based on the use of 2DG, an inhibitor of glycolysis rather than mitochondrial oxidative phosphorylation. We therefore tested the effect of 2DG in *TSC2*$^{-/-}$ versus *TSC2*$^{+/+}$ MEFs by treating these cells for increasing times with this inhibitor. Although basal levels of S6K1 T389 phosphorylation were higher in TSC2-deficient cells, in both cases 2DG led to a similar time-dependent inhibition of S6K1 T389 phosphorylation, a response which closely paralleled the induction of AMPK T172 phosphorylation (Figure 14). Moreover, as seen with oligomycin (Figure 13), the inhibition of S6K1 T389 phosphorylation began to recover by 60 min, which is paralleled by the decrease in AMPK T172 phosphorylation (Figure 14). This transient decrease in S6K1 T389 phosphorylation with both oligomycin and 2DG was highly reproducible and was also observed in A549 cells (see 2DG time course in A549 with matching AMP:ATP ratios and AMPK in vitro kinase activity in Appendix 2) as well as in HEK293 cells (data not shown). This recovery in S6K1 T389 phosphorylation could reflect the cell's adaptation to inhibition of its energy source such that it gradually shifts to the alternative source of energy–glycolysis when oligomycin is used and oxidative phosphorylation when 2DG is used–or could simply reflect the high metabolic turnover of these drugs. We therefore hypothesized that, if this recovery is attributable to a shift in energy sources, the re-addition of a fresh aliquot of the same inhibitor during the phase of recovery would have little to no effect on S6K1 T389 phosphorylation. The results of such an experiment are presented in Figure 15A for oligomycin and Figure 15B for 2DG. We found that, following the recovery of the S6K1 T389 signal at 60 min with either inhibitor, the re-addition of the same inhibitor had little to no effect on S6K1. However, the addition of the other inhibitor dramatically abrogated S6K1 T389 phosphorylation (Figure 15A, B). Taken together, these findings indicate that the mTOR Complex1 signaling pathway does not require the TSC1/2 complex to respond to oligomycin- and 2DG-induced acute energy depletion, and that the cell adapts to compensate for this energy crisis by up regulating alternative energy sources.

The high concentration of 2DG used in our studies (Figures 13, 14) has been shown to induce an osmotic shock as compared to another carbohydrate, mannitol (Inoki, Zhu et al. 2003). This suggests that the effect that we observe with high concentrations of 2DG on mTOR Complex1 signaling could be due to a mixed response of energy depletion and 2DG acting as an osmolite (Inoki, Zhu et al. 2003). We therefore investigated the response of S6K1 T389 phosphorylation with increasing concentrations

A



B

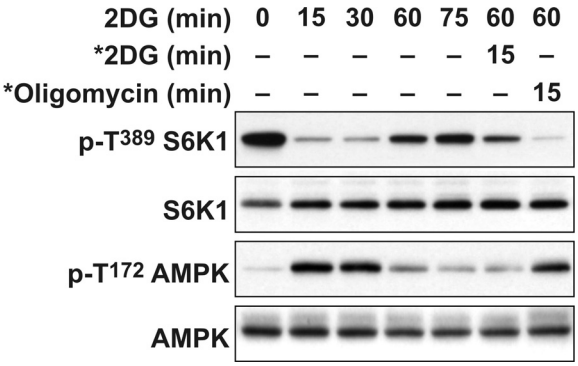


Figure 15: Exponentially growing *TSC2*$^{+/+}$ MEFs treated with **A)** 10 μM oligomycin or **B)** 100 mM 2DG for the times indicated. (*) Denotes the addition of a fresh aliquot of either ATP-depleting agent following a 60 min incubation with the initial inhibitor.

of 2DG in *TSC2*$^{+/+}$ and *TSC2*$^{-/-}$ MEFs (Figure 16). Consistent with Figures 14 and 15B, 100 mM 2DG readily inhibited S6K1 T389 phosphorylation in both cell lines. However concentrations of 25 and 10 mM 2DG, which are known not to have osmotic effects (Inoki, Zhu et al. 2003), showed some resistance in *TSC2*$^{-/-}$ MEFs (Figure 16). This protection seen with lower 2DG concentrations in TSC2-deficient cells led Inoki et al. to conclude that TSC2 was required for the 2DG-induced energy-depletion response of the mTOR Complex1 signaling pathway. However, in contrast to 2DG, treatment of these cells with oligomycin did not result in any difference in the kinetics of S6K1 T389 dephosphorylation (Figure 13), suggesting that the discrepancy between these two ATP-depleting agents might reside in the mechanism of inhibition of ATP production. 2DG inhibits the first step of glycolysis by competing with glucose for its phosphorylation by hexokinase. However, unlike glucose, phosphorylated 2DG cannot be further metabolized to generate ATP and acts as a sink for hexokinase. This effect, and also the fact that hexokinase continues to phosphorylate and to consume energy, effectively reduces the levels of ATP in the cell. Interestingly, TSC2-deficient MEFs have been shown to have elevated levels of Hypoxia Inducible Factor 1 α (HIF1α) (Brugarolas, Vazquez et al. 2003) which, in turn, induces the transcription of glycolytic genes such as hexokinase (Seagroves, Ryan et al. 2001) (Kim, Gao et al. 2007). A feature observed in many cancer cells is a tendency to produce their ATP through glycolysis despite respiration operating normally, a phenomenon known as the “Warburg effect” or aerobic glycolysis (Warburg 1956). Although glycolysis produces much less ATP per molecule of glucose, cancer cells compensate for this inefficiency by increasing the rate of glycolysis through the upregulation of enzymes involved in the catabolism of glucose such as hexokinase II (Dang and Semenza 1999; Pfeiffer, Schuster et al. 2001). Consistent with this observation, many cancer cells have been reported to express high levels of hexokinase II (Pedersen, Mathupala et al. 2002). Since TSC2-deficient cells have been reported to have high levels of HIF1α, we examined the possibility that *TSC2*$^{-/-}$ MEFs might upregulate the expression of hexokinase II. The result of this analysis showed that *TSC2*$^{-/-}$ cells have about a 2-fold increase in hexokinase II protein levels when normalized to tubulin (Figure 17). This observation would explain why *TSC2*$^{-/-}$ MEFs exhibited a protection in S6K1 T389 dephosphorylation at lower doses of 2DG (Figure 16), consistent with their need for higher doses of 2DG to affect the phosphorylation of S6K1 T389. Taken together, these results argue that, like oligomycin, 2DG is capable of reducing the levels of S6K1 T389 phosphorylation in *TSC2*$^{-/-}$ MEFs, and that the

observed resistance to dephosphorylating S6K1 T389 is due to an increased expression of its inhibitory target, hexokinase II.

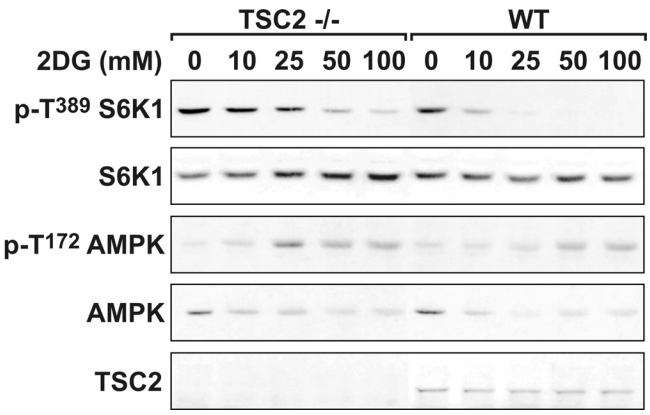


Figure 16: Exponentially growing *TSC2*$^{-/-}$ and *TSC2*$^{+/+}$ MEFs treated for 30 min with 2DG at the indicated concentrations.

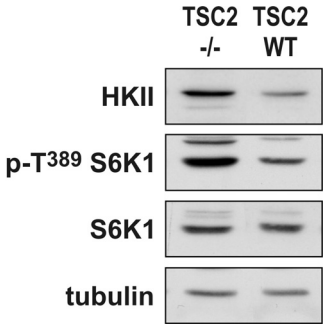


Figure 17: Exponentially growing *TSC2*$^{-/-}$ and *TSC2*$^{+/+}$ MEFs. HKII indicates an immunoblot against hexokinase II.

3. AMPK can signal to mTOR Complex1 independently of TSC2

The findings described above show that there is a good correlation between the phosphorylation of AMPK T172 and the dephosphorylation of S6K1 T389, consistent with the idea that the ATP-depleting agents 2DG and oligomycin blunt mTOR Complex1 signaling through AMPK in a TSC1/2-independent mechanism. In agreement with this model, increasing the concentration of 2DG leads to a dose-dependent increase in AMPK T172 phosphorylation that is closely paralleled by the phosphorylation of acetyl-CoA carboxylase (ACC), a bona fide substrate of AMPK, and the induction of S6K1 T389 dephosphorylation (Figure 18). Because we ruled out TSC1/2 as a mediator of the energy-deprivation response of mTOR Complex1, we wished to assess the extent of AMPK involvement in a situation where TSC2 was absent. We tested whether the activation of AMPK was sufficient to downregulate S6K1 T389 in *TSC2*$^{-/-}$ MEFs by using 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR). This substance is metabolized by the cell into ZMP, an analogue of AMP, and has been shown to directly activate AMPK (Corton, Gillespie et al. 1995). Consistent with previous reports showing that AICAR inhibits mTOR Complex1 in various cell lines (Kimura, Tokunaga et al. 2003), AICAR treatment inhibited basal and insulin-stimulated S6K1 T389 phosphorylation in *TSC2*$^{+/+}$ MEFs (Figure 19). Moreover, this inhibition correlated with the increase in AMPK T172 and ACC S79 phosphorylation, the critical residue phosphorylated by AMPK which inactivates the fatty acid synthesis function of ACC (Ha, Daniel et al. 1994) (Figure 19). Despite *TSC2*$^{-/-}$ MEFs having constitutively high levels of S6K1 T389 phosphorylation, AICAR still induced AMPK T172 and ACC S79 phosphorylation in the absence or presence of insulin, while inhibiting S6K1 T389 phosphorylation (Figure 19). These results support the idea that AMPK activation by ATP-depleting agents could be sufficient to inhibit S6K1 T389 phosphorylation independent of the TSC1/2 complex (see Figures 10-14). To further confirm the implication of AMPK in the mTOR Complex1 signaling pathway, we ectopically expressed constitutively active alleles of the γ regulatory subunit of AMPK which have been shown to be largely independent of any changes in the AMP:ATP ratio (Adams, Chen et al. 2004). These studies showed that the constitutively active mutants R70Q and R152Q induced AMPK T172 activation and were sufficient to affect the mTOR Complex1 signaling pathway (Figure 20). Indeed, overexpression of the wild-type AMPK

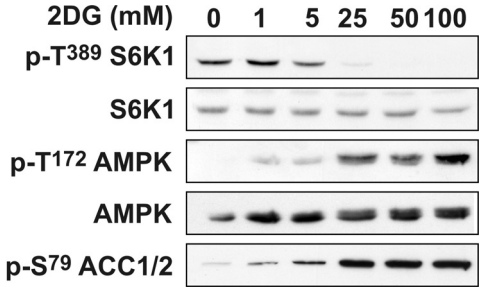


Figure 18: Exponentially growing HEK293 cells treated for 30 min at the indicated concentrations of 2DG.

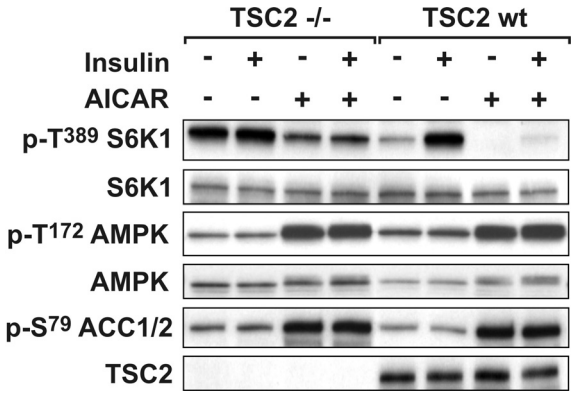


Figure 19: *TSC2*$^{-/-}$ and *TSC2*$^{+/+}$ MEFs were deprived of serum overnight and then stimulated with 200 nM insulin for 30 min. Cells were treated with 2 mM AICAR for 2 h or pretreated for 1.5 h before insulin stimulation.

also led to AMPK T172 phosphorylation and S6K1 T389 dephosphorylation, although to a lesser extent than the constitutively active mutants (Figure 20). Taken together, these studies showed that overexpression of AMPK is sufficient to downregulate mTOR Complex1 signaling.

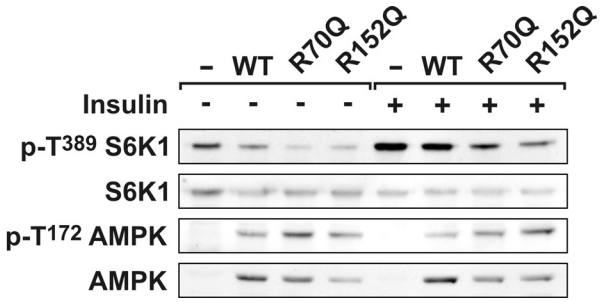


Figure 20: WT indicates that α, β and γ subunits of AMPK were co-expressed in HEK293 cells for 48 h. The R70Q and R172Q indicates that the WT γ subunits were substituted with these respective γ mutants. Following 24 h of ectopic expression, cells were deprived of serum overnight and stimulated with 200 nM insulin for 30 min.

4. AMPK-specific signal to mTOR Complex1 is distinct from general energy depletion

The results obtained above highlight the central role of AMPK in mediating the acute energy depletion signal to mTOR Complex1. However, the importance of AMPK function has been demonstrated mainly through experiments involving long-term treatments with AICAR or ectopic expression of constitutively active AMPK mutants. This raises the possibility that the effect of AMPK on mTOR Complex1 might be indirect. To clarify this point, we closely examined the response of S6K1 T389 phosphorylation relative to the activation of AMPK in conditions of general ATP depletion induced by 2DG and by artificially manipulating the cell's AMP:ATP ratio with AICAR. To allow comparison of these two types of AMPK activators, use was made of C2C12 cells, a non-transformed murine myoblast cell line which has been shown to respond to both AICAR and ATP-depleting agents (Jakobsen, Hardie et al. 2001; Niesler, Myburgh et al. 2007). Unexpectedly, kinetic analysis of AICAR in these cells showed that there was a delay of approximately 30 to 60 min between the induction of AMPK T172 and ACC S79 phosphorylation, as compared to S6K1 T389 dephosphorylation (Figure 21A). This observation suggested that the effect of AMPK on mTOR Complex1 may be indirect, potentially requiring intermediate signaling events or possibly alterations of the transcriptional profile (Leclerc, Viollet et al. 2002). In contrast to AICAR, however, a delay in the response did not occur with 2DG treatment. Rather, the increase in AMPK T172 correlated well with S6K1 T389 dephosphorylation (Figure 21B), which highlights a discrepancy between the effect of AICAR and 2DG. Indeed, these results show that, despite the finding that AICAR treatment activated AMPK by 30 min; S6K1 T389 phosphorylation was not affected (Figure 21A). Taken together, these findings suggest that S6K1 dephosphorylation induced by the activation of AMPK is not equal to S6K1 dephosphorylation by general ATP depletion.

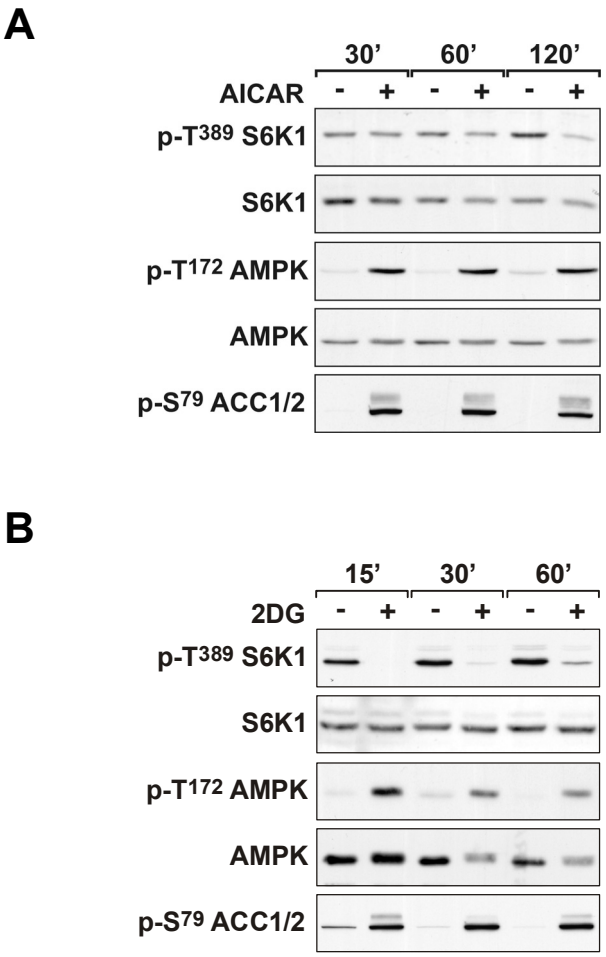


Figure 21: Exponentially growing C2C12 myoblasts treated either with **A)** 2 mM AICAR or **B)** 100 mM 2DG for the indicated times.

5. Energy depletion inhibits mTOR Complex1 signaling independently of LKB1 and AMPK

Since these studies were largely based on correlative evidence, we questioned the role of AMPK in mediating the acute energy depletion signal to the mTOR Complex1 signaling pathway. To address further the question of whether AMPK is required for the energy depletion signal to mTOR Complex1, we subjected MEFs deficient for both catalytic subunits of AMPK (Laderoute, Amin et al. 2006) to increasing concentrations of 2DG. As anticipated, *AMPKα1*$^{+/+}$/*AMPKα2*$^{+/+}$ MEFs showed a dose-dependent decrease in S6K1 T389 phosphorylation correlating with an increase in AMPK T172 and ACC S79 phosphorylation (Figure 22). Surprisingly, however, 2DG induced the dephosphorylation of S6K1 T389 even in the absence of any functional AMPK when compared to their wild-type counterpart (Figure 22). Moreover, unlike *TSC2*$^{-/-}$ MEFs, no resistance was observed in *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ MEFS at lower concentrations of 2DG, supporting the idea that the defect seen in the *TSC2*$^{-/-}$ MEFs is TSC2-specific, potentially linked to the elevated expression of hexokinase II (Figures 16, 17). Given the acute inhibition of mTOR Complex1 by 2DG in AMPK α_1/α_2- deficient MEFs, we asked whether oligomycin would produce the same effect and whether AICAR would still have an inhibitory effect on mTOR Complex1 signaling. The results of this experiment showed that both agents induced AMPK T172 and ACC S79 phosphorylation in *AMPKα1*$^{+/+}$/*AMPKα2*$^{+/+}$ MEFs, which was paralleled by S6K1 T389 dephosphorylation (Figure 23A). However, despite AICAR's stronger effect on AMPK T172 and ACC S79 phosphorylation, compared to oligomycin, the decrease in S6K1 T389 phosphorylation was more pronounced with the ATP-depleting agent (Figure 23A). In *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ cells, oligomycin still blocked insulin induction of S6K1 T389 phosphorylation supporting the idea that, in the absence of AMPK, mTOR Complex1 sensitivity is not exclusive to 2DG treatment, but probably more generally related to ATP deprivation status. However, AICAR treatment had no effect on S6K1 T389 phosphorylation in this cell line suggesting that the effect of AICAR on mTOR Complex1 is essentially mediated by AMPK (Figure 23A). Moreover, no detectable AMPK T172 or ACC S79 phosphorylations were observed with either oligomycin or AICAR treatment, validating the complete loss of any functional alleles of AMPK in this double knock-out cell line (Figure 23A). To confirm that these effects were

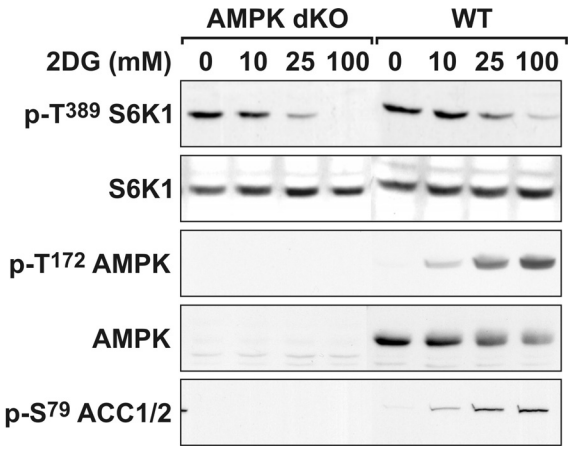


Figure 22: Exponentially growing primary *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ (AMPK dKO) and *AMPKα1*$^{+/+}$/*AMPKα2*$^{+/+}$ MEFs (WT) treated with 2DG for 30 min at the indicated concentrations.

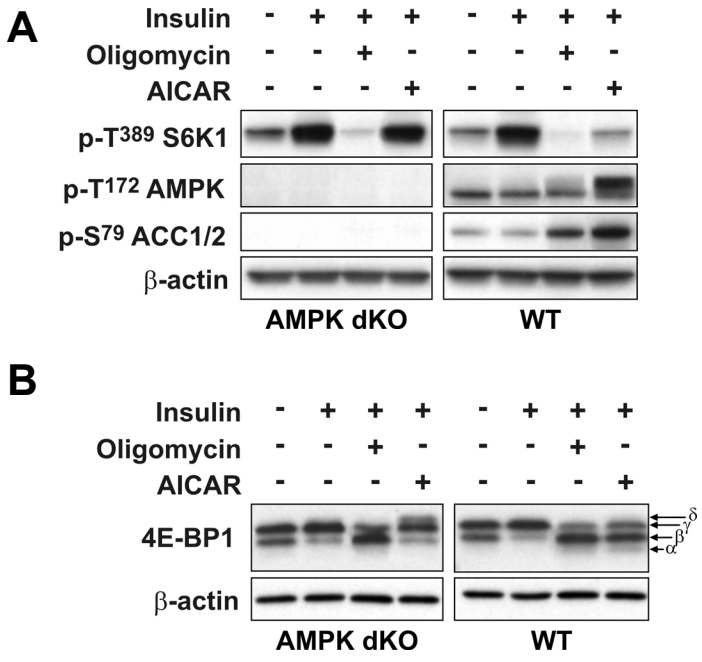


Figure 23: **A,B)** Exponentially growing immortalized *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ (AMPK dKO) and *AMPKα1*$^{+/+}$/*AMPKα2*$^{+/+}$ MEFs (WT) were deprived of serum overnight and then either stimulated for 30 min with 200 nM insulin alone or co-treated with 10 μM oligomycin and insulin for 30 min or pretreated with 2 mM AICAR for 1.5 h before insulin stimulation.

mediated through mTOR Complex1, we examined another well described substrate of mTOR Complex1 signaling, 4E-BP1. We found that in both wild-type and *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ cells, the electrophoretic migration of 4E-BP1 was decreased with insulin stimulation and that this shift was eliminated with oligomycin treatment (Figure 23B). In contrast, pretreatment with AICAR abolished this shift only in *AMPKα1*$^{+/+}$/*AMPKα2*$^{+/+}$ MEFs and not in the AMPK-deficient MEFs, consistent with the protection observed earlier for S6K1 T389 phosphorylation (Figure 23B). To explore these observations further, we tested whether the major upstream activating kinase of AMPK, LKB1 (Hawley, Boudeau et al. 2003; Woods, Johnstone et al. 2003) would be required for the ATP depletion response to mTOR Complex1. *LKB1*$^{-/-}$ MEFs were used to test this response, which showed that treatment of these cells with oligomycin had no effect on AMPK T172 and ACC S79 phosphorylations, consistent with LKB1 acting as the upstream kinase for AMPK (Figure 24). However, oligomycin was still able to induce the inhibition of insulin induced S6K1 T389 phosphorylation (Figure 24). Surprisingly, AICAR could still activate AMPK, as assessed by the increase in AMPK T172 and ACC S79 phosphorylation, circumventing the need for LKB1. However, the activation of AMPK was only accompanied with a slight decrease in S6K1 T389 phosphorylation (Figure 24). Taken together, these results suggest that the LKB1-AMPK axis is not required for the acute energy deprivation response to mTOR Complex1 and argue for the existence of an alternative pathway distinct from that of the LKB1-AMPK axis, which is involved in regulating the energy depletion.

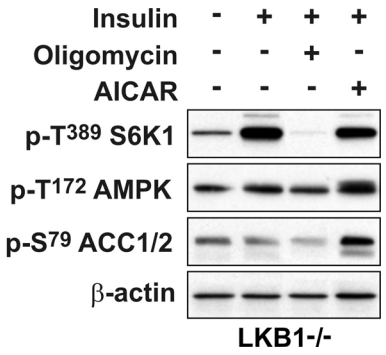


Figure 24: Exponentially growing immortalized *LKB1*$^{-/-}$ MEFS were deprived of serum overnight and then either stimulated for 30 min with 200 nM insulin alone or co-treated with 10 μM oligomycin and insulin for 30 min or pretreated with 2 mM AICAR for 1.5 h before insulin stimulation.

6. Regulation of mTOR Complex1 signaling by chronic energy depletion

So far, all our studies were based on short term or acute inhibition of mTOR Complex1. However, mTOR Complex1 has also been shown to respond to long term or chronic energy depletion. This response is thought to be mediated by REDD1 through the TSC1/2 complex (Sofer, Lei et al. 2005). Moreover, the role of AMPK activation in the chronic response has been questioned since these authors could not rule out AMPK's implication in the regulation of REDD1 function. This is because dominant-interfering alleles of AMPK prevented the inhibition of mTOR Complex1 signaling in long-term 2DG treatments suggesting that AMPK might be required for this effect (Sofer, Lei et al. 2005). To assess the role of AMPK in the chronic energy depletion response to mTOR Complex1 signaling, we assessed S6K1 T389 phosphorylation over time during 2DG treatment of wild-type and AMPK double knock-out MEFs. We found that S6K1 T389 phosphorylation responded acutely to 2DG in both genotypes (Figure 25). Similar to previous results (Figure 15B), this inhibition was transient and recovered completely by 2 hours (Figure 25). However, chronic treatment with 2DG abrogated S6K1 T389 phosphorylation in both cell types consistent with the notion that induction of REDD1 at these later time points would downregulate mTOR Complex1 signaling (Figure 25). That 2DG affected mTOR Complex1 signaling irrespective of AMPK status suggested that contrary to what was expected (Sofer, Lei et al. 2005), AMPK is not required for the chronic energy depletion response. We also observed that AMPK T172 phosphorylation was transient in *AMPKα1*$^{+/+}$/*AMPKα2*$^{+/+}$ MEFs, as seen previously in Figure 15B and 21B. Nonetheless, ACC S79 phosphorylation was maintained for at least 8 hours. An identical time-course experiment, but using oligomycin treatment, showed a comparable trend in S6K1 T389 and ACC S79 phosphorylation (data not shown). Together, these results support a model whereby AMPK is not required for the chronic energy depletion response.

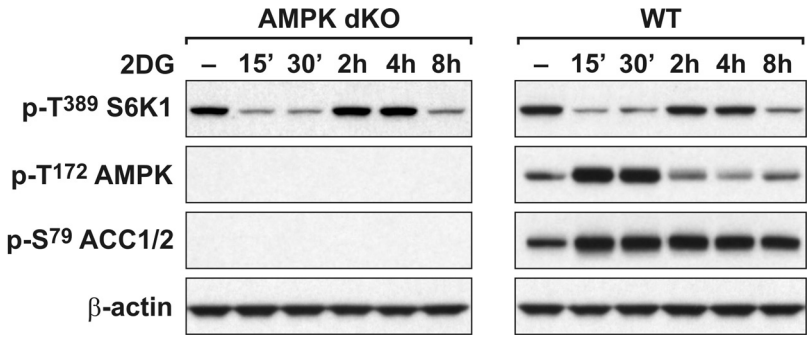


Figure 25: Exponentially growing immortalized *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ (AMPK dKO) and *AMPKα1*$^{+/+}$/*AMPKα2*$^{+/+}$ MEFs (WT) treated with 100 mM 2DG for the indicated times.

To substantiate the relevance of the pharmacological approaches used in our studies, we wished to assess the role of AMPK by using a more physiologically relevant energy deprivation paradigm, such as glucose deprivation. To do this, we starved *AMPKα1*$^{+/+}$/*AMPKα2*$^{+/+}$ and *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ MEFs for glucose at increasing times and assessed the role of AMPK in this starvation response as well in the glucose re-feeding response. We found that in both cell types, S6K1 T389 phosphorylation was substantially inhibited at 2 hours of glucose starvation and, as seen previously with 2DG and oligomycin (Figures 13-15), this decrease started to recover at later times in *AMPKα1*$^{+/+}$/*AMPKα2*$^{+/+}$ MEFs (Figure 26). However, there was no recovery of S6K1 T389 phosphorylation in *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ MEFs at those times. In fact, the signal seemed to diminish even further, suggesting that AMPK is not required to mediate the glucose deprivation signal to mTOR Complex1. Moreover, the response to glucose stimulation following 8 hours starvation in both cell types did not reveal any defect in these cells suggesting that AMPK does not play a role in either the starvation response or the glucose stimulation response. Together, these results suggest that like 2DG, glucose starvation induced chronic energy depletion response to mTOR Complex1 does not require AMPK.

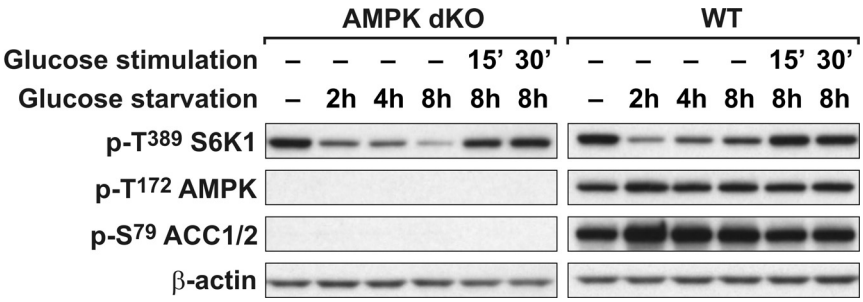


Figure 26: Exponentially growing immortalized *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ (AMPK dKO) and *AMPKα1*$^{+/+}$/*AMPKα2*$^{+/+}$ MEFs (WT) were deprived of serum and glucose for the time indicated and then stimulated with 1X glucose for 15 or 30 min.

7. Metformin inhibits mTOR Complex1 signaling independently of AMPK and TSC2

Metformin is the most commonly prescribed oral medication for treatment of type II *diabetes mellitus*. It has been shown to lower hyperglycemia by decreasing gluconeogenesis in the liver and enhancing glucose uptake in skeletal muscle (Zhou, Myers et al. 2001). Activation of AMPK has been reported to be required for metformin's ability to decrease glucose production by hepatocytes (Zhou, Myers et al. 2001). Moreover, this antidiabetic drug has also been shown to suppress mTOR Complex1 signaling, which is thought to be mediated by AMPK (Shaw, Lamia et al. 2005). Despite extensive studies on metformin, there are still uncertainties regarding its mechanism of action. It is believed that AMPK activation is achieved through the ability of metformin to inhibit mitochondrial complex I (Owen, Doran et al. 2000) (Leverve, Guigas et al. 2003). Since oligomycin is also a mitochondrial inhibitor, and on the basis of our finding that it inhibits mTOR Complex1 independently of AMPK (Figure 23), we wished to test whether metformin could affect mTOR Complex1 independently of AMPK. In agreement with our previous results, we found that in wild-type cells metformin led to an increase in AMPK T172 and ACC S79 phosphorylation, whereas it abrogated S6K1 T389 phosphorylation (Figure 27A). Surprisingly, treatment of *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ MEFs with metformin also inhibited S6K1 T389 phosphorylation (Figure 27A), unlike AICAR (Figure 23). Moreover, in the absence of AMPK there was no effect on ACC S79 phosphorylation (Figure 27A), supporting the hypothesis that metformin, like oligomycin, can operate through an AMPK-independent mechanism to inhibit mTOR Complex1 signaling. The effect seen on S6K1 T389 requires metformin incubation over twenty-four hours due to its slow permeation and accumulation in the mitochondrial matrix (Owen, Doran et al. 2000) (Hardie 2006). Thus, it could be argued that the observed effects are not acute but, rather, secondary. To address this issue we turned to phenformin, an analog of metformin, which has also been shown to inhibit mitochondrial complex I, but due to its higher lipophilic properties, accumulates much rapidly in the mitochondrial matrix and therefore exerts its effect with shorter incubation times (Owen, Doran et al. 2000). Phenformin treatment of wild-type MEFs showed that 1h incubation was sufficient to increase AMPK T172 and ACC S79 phosphorylations and, like merformin, S6K1 T389 phosphorylation was abolished (Figure 27B). In contrast, phenformin had no effect on

ACC S79 phosphorylation in *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ MEFs, but still abrogated S6K1 T389 phosphorylation (Figure 27B). Taken together, phenformin like metformin can downregulate S6K1 T389 phosphorylation independently of the AMPK status.

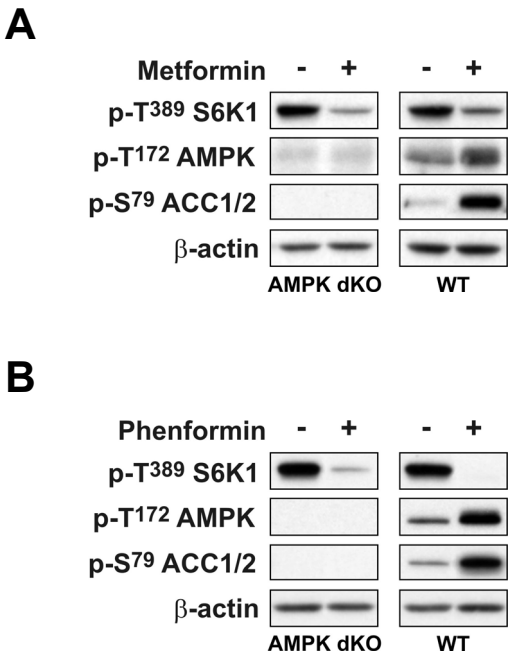


Figure 27: Exponentially growing immortalized *AMPKα1*$^{-/-}$/*AMPKα2*$^{-/-}$ (AMPK dKO) and *AMPKα1*$^{+/+}$/*AMPKα2*$^{+/+}$ MEFs (WT) treated either with **A)** 10 mM metformin for 24 h or **B)** 6 mM phenformin for 1 h.

In agreement with the model that 2DG and oligomycin-induced energy deprivation-mediated mTOR response is independent of TSC2, we also observed that metformin and phenformin abolished S6K1 T389 phosphorylation. This finding confirmed that, like oligomycin, these antidiabetic drugs act independently of the AMPK-TSC2 axis to inhibit mTOR Complex1 (Figure 28A,B). Altogether, these findings reveal the existence of a novel signaling pathway that regulates mTOR Complex1 signaling in response to acute energy depletion, which is independent of those pathways utilized by either TSC1/2 and/or AMPK (Figure 29).

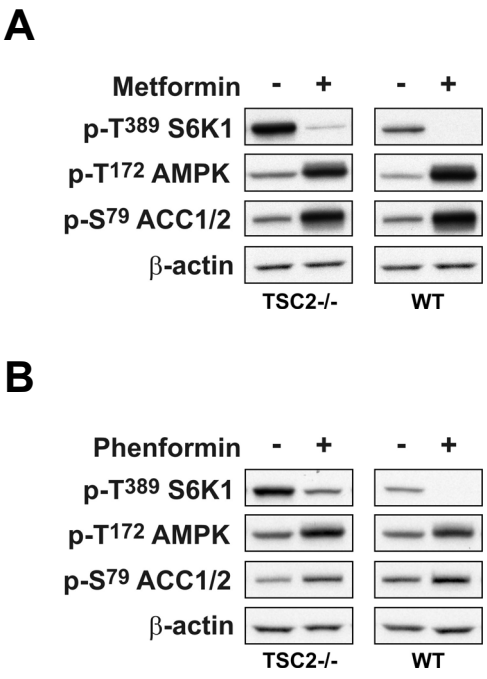


Figure 28: Exponentially growing *TSC2*$^{-/-}$ and *TSC2*$^{+/+}$ MEFs treated either with **A)** 10 mM metformin for 24 h or **B)** 6 mM phenformin for 1 h.

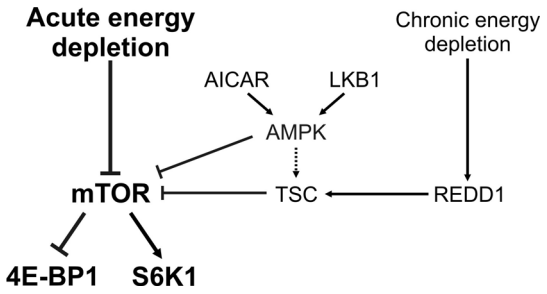


Figure 29: These studies revealed the existence of an autonomous acute energy dependent mTOR Complex1 signaling which acts independently of TSC1/2 complex and AMPK. This novel signaling pathway is also thought to be used by the antidiabetic drug metformin.

8. Search for a mediator of the energy deprivation signal to mTOR Complex1

Having found that the LKB1-AMPK-TSC2 axis is not required for mediating the acute energy deprivation signal to mTOR Complex1, we looked for possible candidates that could impact on mTOR Complex1. One obvious candidate to test was the human class 3 PI3K or hVps34 which was recently shown to mediate the nutrient signals to mTOR Complex1 (Byfield, Murray et al. 2005; Nobukuni, Joaquin et al. 2005). In these studies, hVps34 activity was shown be sensitive to glucose starvation and oligomycin treatment (Byfield, Murray et al. 2005). We wished to look into this possibility by analyzing the in vitro kinase activity of hVps34 following 2DG treatment as well as glucose starvation in HEK293 cells. The results of such experiment showed that none of these treatments affected the in vitro kinase activity of hVps34 as assessed by the production of PI(3)P, the product of hVps34 activity (Figure 30). We also failed to observe any effects on hVps34 activity in response to 2DG and glucose starvation in other cell lines (data not shown).

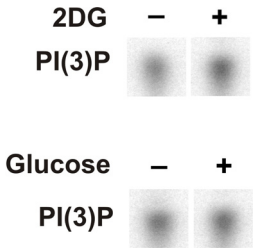


Figure 30: Top panel, exponentially growing HEK293 cells treated with 100 mM 2DG for 30 min. Bottom panel, HEK293 cells were deprived of serum overnight and then further straved for glucose for 2h.

Although we were unable to observe any changes in the in vitro hVps34 activity in response to 2DG and glucose starvation, we investigated further to measure the PI(3)P levels by immunocytochemistry as described in earlier studies (Nobukuni, Joaquin et al. 2005). Unfortunately, we could not see any changes in the PI(3)P staining

upon 2DG and glucose starvation (data not shown). Taken together, these experiments suggest that hVps34 may not be a critical mediator of the energy deprivation signal to mTOR Complex1.

mTOR Complex1 activity has been shown to be sensitive to a redox sensitive mechanism (Sarbassov and Sabatini 2005). The oxidizing reagent phenylarsine oxide (PAO) induces disulfide bonds of thiol groups of cysteine residues in proteins and pre-treatment with this reagent has been shown to protect mTOR Complex1 from the effect of 2DG (Sarbassov and Sabatini 2005). We tested the ability of 2DG and oligomycin to affect S6K1 T389 phosphorylation of cells pretreated with PAO. The result of this experiment shows that PAO increases basal S6K1 T389 phosphorylation, however, the energy depleting agents still affected S6K1 T389 phosphorylation to the same extent as without PAO (Figure 31). This suggested that the mechanism by which the ATP depleting agents affect mTOR Complex1 does not involve a redox change. Although Sarbassov et al. obtained identical results to ours, they concluded that a redox-based signaling mechanism operates to regulate mTOR Complex1. Given the importance of mTOR Complex1 signaling in diabetes and cancer, it will be critical to elucidate the molecular mechanism by which acute energy deprivation controls mTOR Complex1 activation in future studies (see Discussion).

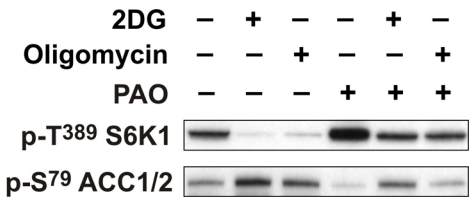


Figure 31: Exponentially growing HEK 293 cells were either pre-treated with 5 μM of PAO or not, followed by treatment with 100 mM 2DG and 10 μM oligomycin for 30 min.

V. DISCUSSIONS

1. mTOR Complex1 signaling, cellular energy & mitochondrial metabolism

Initial studies establishing the relationship between mTOR and cellular energy status showed that ATP depleting agents, such as the glycolytic inhibitor 2-deoxyglucose (2DG) and the mitochondrial electron transport chain inhibitor rotenone, affected mTOR signaling to a similar extent to which they depleted ATP levels in the cell (Dennis, Jaeschke et al. 2001). Our results confirm the existence of this relationship in the presence of 2DG (Figure 1). Moreover, the importance of energy status to mTOR Complex1 signaling is highlighted by the fact that energy deprivation signals are dominant over hormone and nutrient signals, as 2DG and the mitochondrial inhibitor oligomycin abolished the stimulation of S6K1 T389 phosphorylation by insulin and amino acids (Figures 6, 7). Mitochondrial oxidative phosphorylation is the most efficient process to produce ATP in the cell, however many cancer cells shift to aerobic glycolysis, despite having a normally operating mitochondrial respiration system (Warburg 1956). Many cultured cell lines are derived from tumors and exhibit this feature despite being maintained in normoxic conditions (Assaily and Benchimol 2006). However, there is no consensus as to whether cultured cell lines are exclusively dependent on glycolysis, since many cell lines have been demonstrated to rely equally on both processes to generate their energy needs (Sariban-Sohraby, Magrath et al. 1983). We observe that HEK293 cells display a higher sensitivity to inhibition of S6K1 T389 phosphorylation when treated with 2DG as opposed to oligomycin, suggesting that these cells might be more dependent on glycolysis for the production of ATP (Figure 2A). In contrast, these inhibitors are equally potent in decreasing S6K1 T389 phosphorylation in MEFs and FL5.12 cells (Figures 3A and 5A), indicating that they may be differently dependent on glycolysis versus mitochondrial respiration, as compared to HEK293 cells. Nevertheless, these observations confirm that despite these cell lines having different rates of glycolysis and mitochondrial respiration, both sources are being utilized for their energy needs.

Although experiments performed with 2DG showed that the decrease in S6K1 T389 phosphorylation correlated with the ATP levels in the cell, oligomycin failed to

show a tight relationship between such levels and S6K1 T389 phosphorylation. This inhibitor reproducibly induced only a modest reduction in the ATP levels, and seemingly consistent with this a small increase in the AMP:ATP ratio, regardless of the cell type tested, suggesting that this was not a cell type specific issue (Figures 2B,C and 3B, C). Nevertheless, oligomycin had a pronounced effect on the decrease in S6K1 T389 phosphorylation (Figures 2A and 3A). A comparative dose response between 2DG and oligomycin inhibition of S6K1 T389 phosphorylation supported the notion that the S6K1 T389 signal could be decoupled from total ATP levels in the cell (Figure 4). Although this was unexpected given the known inhibitory effect of oligomycin (Boyer 1997; Johnson, Cleary et al. 2006), the ATP measurements following treatments with oligomycin have also been inconsistent when comparing findings in the literature, even within the same human cell lines (Leist, Single et al. 1997; Karawajew, Rhein et al. 2005). In addition, the measurements of total ATP levels do not take into account its cellular localization. Therefore, it is possible that the absence of significant changes in ATP measurements observed with oligomycin could be due to local variations of ATP levels. To circumvent this problem, one should potentially measure the rate at which ATP is produced by mitochondria, or alternatively enrich mitochondria by fractionation to identify changes in ATP emanating from this cellular compartment. Given the limitation of the technique and what is known about the mode of action of oligomycin, it is quite reasonable to speculate that the mechanism by which 2DG and oligomycin affect mTOR Complex1 signaling is through depletion of ATP. To support the role of 2DG and oligomycin in the ATP depletion response, we measured the mitochondrial membrane potential ($\Delta\Psi_m$). 2DG and oligomycin have different cellular targets, with 2DG targeting the first step of glycolysis -hexokinase- and oligomycin inhibiting the proton channel of the F_0 segment of the ATP synthetase complex. Our experiments showed that $\Delta\Psi_m$ was altered in opposite directions that is, 2DG increased $\Delta\Psi_m$ whereas oligomycin decreased $\Delta\Psi_m$ as expected (Figure 5). This change in $\Delta\Psi_m$ would result in a reduction of ATP production and indicates that oligomycin treatment was effectively operating on its target to affect mitochondrial oxidative phosphorylation.

An unexpected finding revealed by our studies was the apparent switching of energy sources to mediate mTOR Complex1 signaling as a function of the energy depleting agent. When cells are treated with oligomycin, ATP production, and thus mTOR Complex1 signaling, appears to become solely dependent on glycolysis. Readdition of oligomycin has little to no effect on S6K1 T389 phosphorylation, but the

cells retain their sensitivity to the glycolytic inhibitor 2DG (Figure 15A). Indeed, measurements of ATP levels and AMP:ATP ratios, showed that the addition of 2DG under these conditions causes a pronounced drop in the ATP levels and a dramatic rise in the AMP:ATP ratio (> 250-fold) (see Appendix 3). Conversely, where an initial treatment with 2DG was followed by oligomycin a similar phenomenon was observed, an apparent switch in the energy source as scored by mTOR Complex1 signaling (Figure 15B). From these studies we hypothesized that at the starting point, mTOR Complex1 signaling is sensitive to both energy sources, as shown by the ability of either the glycolytic inhibitor 2DG or the mitochondrial respiratory inhibitor oligomycin to block mTOR Complex1 signaling. However, when one energy source is inhibited, the cell appears to adjust the energy status by upregulating the alternative source of energy. This apparent compensatory mechanism may represent a fundamental protective response that all cells exhibit in order to cope with metabolic extremes. A recent report by Liu et al. showed that the neuronal uncoupling protein 4 (UCP4), a resident of the inner membrane of the mitochondria, modulates an adaptive shift of energy metabolism in response to oxidative stress conditions. Liu et al. showed that oxidative stress induces UCP4 expression, which mediates an increase in glucose uptake and glycolysis to compensate for the reduced mitochondrial ATP production from oxidative phosphorylation (Liu, Chan et al. 2006). UCP4 is predominantly expressed in neurons, whereas UCP2 is widely expressed in multiple tissues (Liu, Chan et al. 2006). It is possible that the adaptive switch that we observe in our studies may be mediated by UCP2. It will be important to determine whether UCP2 is a component of an energy response module linked to mTOR Complex1 signaling. Interestingly, mTOR Complex1 has been shown to be part of a stress-sensing module, which locates to mitochondria and could potentially function to sense glucose deprivation (Desai, Myers et al. 2002).

2. mTOR Complex1 regulation by acute energy depletion

With respect to acute energy depletion, our laboratory initially hypothesized a model by which mTOR was regulated by homeostatic levels of ATP and that mTOR itself would sense the ATP levels due to its apparent high km for ATP (Dennis, Jaeschke et al. 2001). However, later studies suggested that AMPK would serve as the primary sensor of the energy status of the cell and would regulate mTOR Complex1 activity by potentially increasing the GAP activity of TSC2 towards GTP-bound Rheb (Inoki, Zhu et al. 2003). The absence of sequence homology in the *Drosophila* TSC2 region where the consensus AMPK phosphorylation motifs are found in human led us hypothesize that the acute energy depletion signal to dTOR might not be mediated by dTsc1/2 complex in the fly (Figure 9, 10). Indeed, RNA interference studies in which the levels of dTsc1 or dTsc2 were depleted in Kc167 cells, showed that oligomycin treatment still led to an acute decrease in dS6K activity (Figure 11). These observations prompted a similar analysis in mammalian cells, and led us to the finding that neither TSC1 nor TSC2 were required for the acute energy deprivation response with respect to inhibition of mTOR Complex1 signaling (Figures 12-14). Moreover, consistent with earlier observations, these findings showed that the inhibitory response induced by either ATP depleting agent, oligomycin or 2DG, was transient, as the phosphorylation of dS6K or S6K1 began to recover over time in both cases (Figures 10, 13-15).

The studies described here also showed that TSC2-deficient cells exhibited a resistance to inhibition of mTOR Complex1 signaling in the presence of 25mM 2DG, a concentration at which the osmotic effect of 2DG was expected to be negligible as compared to its ability to act as an ATP depleting agent (Inoki, Zhu et al. 2003). We also found that 2DG at concentrations of 25mM or lower, was inefficient in decreasing S6K1 T389 phosphorylation in TSC2 deficient MEFs as compared to their wild type counterpart (Figure 16). However, TSC2 deficient MEFs have been reported to have elevated expression of the Hypoxia Inducible Factor 1 α (HIF1α), which drives the expression of a number of genes required for increasing the rate of glycolysis, such as the inhibitory target of 2DG, hexokinase II (Seagroves, Ryan et al. 2001) (Brugarolas, Vazquez et al. 2003) (Kim, Gao et al. 2007). Recently, it has been reported that increases in the expression of the HIF1α target gene hexokinase II, reduces the effect of 2DG as a chemotherapeutic agent in inhibiting glycolysis in tumors (Maher, Wangpaichitr et al. 2007). Analysis of hexokinase II levels in TSC2 deficient cells

showed that its levels were upregulated as compared to its wild type counterpart (Figure 17). That this is a critical hallmark of TSC2 deficient cells, is consistent with recent reports showing that decreasing the concentration of glucose in the culture media effectively lowers the concentration of 2DG required to inhibit mTOR Complex1 signaling (Smith, Finn et al. 2005); i.e. if less glucose is present, there is less competition for 2DG in binding to hexokinase II. Hence, we conclude that the resistance observed in these cells is most likely due to higher expression levels of one of the targets of this inhibitor, hexokinase II (Figure 17). These findings are also consistent with the observation that we observe no resistance in the dephosphorylation of S6K1 T389 with the mitochondrial inhibitor oligomycin in TSC2 deficient MEFs. Taken together, our findings suggest that TSC1/2 complex is not required for the acute energy depletion response (Figures 11-14).

In agreement with a model where AMPK mediates the acute energy depletion inhibitory effect on mTOR Complex1 signaling (Inoki, Zhu et al. 2003), activation of AMPK by AICAR is sufficient to induce S6K1 T389 dephosphorylation even in the absence of TSC2 (Figure 19). Furthermore, ectopic expressions of constitutively activated alleles of AMPK are sufficient to downregulate mTOR Complex1 signaling (Figure 20). Consistent with these findings, AMPK has been proposed to potentially negatively regulate mTOR Complex1 function by mediating mTOR phosphorylation at the nutrient sensitive residue threonine 2446 (Cheng, Fryer et al. 2004). Although the physiological significance of this phosphorylation site and the role of AMPK in regulating the nutrient-dependent function of mTOR through this site have not been determined, it remained possible that mTOR Complex1 function could be regulated by AMPK in the absence of TSC2. Moreover, protein sequence analysis of the nutrient sensitive component of the mTOR Complex1 function, raptor, revealed that it contains at least one AMPK consensus phosphorylation motif (data not shown), raising the possibility that AMPK may regulate the activity of the mTOR Complex1 by directly phosphorylating a critical component required for its ability to recognize downstream substrates.

Given the findings above, it was unexpected that in C2C12 cells, the 2DG-induced S6K1 T389 dephosphorylation was much more rapid than that induced by AICAR (Figure 21A, B), despite similar effects on ACC S79 phosphorylation. This finding suggested that AMPK mediated S6K1 T389 inactivation may be indirect, leading us to question the role of AMPK in the acute energy depletion response in inhibiting mTOR Complex1 signaling. Further studies using AMPK α_1/α_2 deficient MEFs showed that 2DG

and oligomycin were still able to abrogate S6K1 T389 and 4E-BP1 phosphorylation in the absence of AMPK (Figure 22, 23 and data not shown). Importantly, AICAR failed to inhibit the phosphorylation of S6K1 T389 and 4E-BP1 in these same cells, highlighting the requirement of AMPK for AICAR mediated response (Figure 23). Additionally, no resistance in S6K1 T389 phosphorylation is observed at lower concentrations of 2DG in AMPK α_1/α_2- deficient MEFs supporting the idea that hexokinase II effect is a TSC complex deficient phenomenon (Figures 23, 16). Consistent with this observation, AMPK α_1/α_2- deficient MEFs do not express higher levels of HIF1α under normoxic conditions compared to their wild type counterpart (Laderoute, Amin et al. 2006), supporting a model whereby TSC1/2 complex and AMPK are not required for the acute energy depletion response to mTOR Compex1.

That AMPK was not required for the transduction of the acute energy deprivation signal to mTOR Complex1 was unexpected, since it is well established that AMPK functions in suppressing energy consuming processes during an energy crisis (Carling 2004; Hardie, Hawley et al. 2006). However, initial signs indicating that AMPK was dispensable for the mTOR Complex1 signaling came from the observation, that in contrast to TSC2 deficient cells, AMPK α_1/α_2- deficient MEFs did not show constitutively elevated levels of S6K1 T389 phosphorylation in tonic serum as well as in a serum and glucose starved settings (Figures 22, 23, 25, 26). Again, this was an unexpected observation in AMPK α_1/α_2- deficient MEFs, given the negative regulatory role of AMPK on mTOR Complex1 signaling (Inoki, Zhu et al. 2003). AMPK has been implicated in mitochondrial biogenesis in muscle (Zong, Ren et al. 2002) and, recently hepatocytes from AMPK α_1/α_2- deficient liver have been shown to display reduced rates of oxygen consumption and lower total oxidative capacity, which results in overall lower ATP levels (Guigas, Taleux et al. 2007). In our hands, however, there was no indication of overall lower ATP levels in AMPK α_1/α_2- deficient MEFs. Nevertheless, these observations do not exclude the possibility that *AMPKα1*$^{-/-}$*/AMPKα2*$^{-/-}$ MEFs might have evolved to adapt for the absence of AMPK by replacing its function with other AMPK-like kinases. Indeed, the inspection of the human kinome reveals the existence of 12 protein kinases that are closely related to AMPK α_1 and AMPK α_2 (Lizcano, Goransson et al. 2004). Although no apparent function has been assigned to most of these kinases, it remains possible that given the high sequence similarity of their catalytic subunits, they may function as redundant AMPK α_1/α_2 and could potentially serve as a replacement for AMPK α_1/α_2.

Nonetheless, it should be noted that these kinases would have to compensate for AMPK functions that do not involve the regulation of ACC, since absence of AMPK in the AMPK α_1/α_2- deficient MEFs, completely abolished ACC S79 phosphorylation (Figures 22, 23, 25, 26).

To explore the acute energy deprivation response on mTOR Complex1 signaling further, we tested the requirement of LKB1, a major upstream kinase implicated in regulating AMPK activation (Hawley, Boudeau et al. 2003; Woods, Johnstone et al. 2003). Treatment of LKB1 deficient cells with oligomycin showed that as in AMPK α_1/α_2- deficient MEFs, S6K1 T389 phosphorylation was blocked. However, in contrast to AMPK α_1/α_2- deficient MEFs, AICAR induced a small but significant decrease in S6K1 T389 phosphorylation, which was consistent with the small increase in AMPK T172 and ACC S79 phosphorylation (Figure 24). This effect was most probably due to the intrinsic activation of AMPK by ZMP, the metabolized end product of AICAR and an analogue of AMP. Indeed, as part of the study of the mechanism of activation of AMPK, it was recently suggested that AMP activates AMPK not only allosterically by binding to the regulatory γ subunit but also by protecting T172 from dephosphorylation by phosphatases (Sanders, Grondin et al. 2007). That AMPK was still activated in the absence of LKB1 could also be accounted for the action of other AMPK kinases, such as Ca^{2+}/calmodulin-dependent protein kinase (Hawley, Pan et al. 2005), or the more recently proposed TAK1 (Momcilovic, Hong et al. 2006) and ataxia telangiectasia mutated (ATM) kinases (Sun, Connors et al. 2007). Taken together, our biochemical and genetic data argue that mTOR Complex1 is regulated by acute energy deprivation independently of the LKB1-AMPK-TSC2 axis (see model at Figure 29).

3. mTOR Complex1 regulation during chronic energy deprivation

Previous reports showed that the energy depletion affects mTOR Complex1 temporally in two phases, an acute phase and a late/chronic phase (Dennis, Jaeschke et al. 2001) (Inoki, Zhu et al. 2003) (Liu, Cash et al. 2006) (Sofer, Lei et al. 2005). We also observe both an acute and chronic response with 2DG and oligomycin (Figure 25 and data not shown). The chronic response to energy depletion has been shown to be mediated by the transcriptional upregulation of REDD1. REDD1 has been shown to negatively impact on mTOR Complex1 signaling in a TSC1/TSC2 dependent manner, however, the mechanism by which energy depletion induces REDD1 and how REDD1 protein conveys the energy signal through TSC1/TSC2 to suppress mTOR Complex1 signaling is not known (Brugarolas, Lei et al. 2004) (Sofer, Lei et al. 2005). In this context, the role of AMPK in the chronic energy deprivation response has been somewhat unclear. Indeed, ectopic expression of a dominant-interfering allele of AMPK (DN-AMPK) was sufficient to protect mTOR Complex1 signaling from the long term effects of 2DG induced inhibition. However, the DN-AMPK could not by-pass the negative effect of ectopic expression of REDD1 on mTOR Complex1 signaling, suggesting that AMPK lies upstream of REDD1. However, the authors could not detect any modifications or stabilization of the REDD1 protein, nor could they detect a block in REDD1 induction when DN-AMPK was expressed following energy stress (Sofer, Lei et al. 2005). These findings led them to suggest that REDD1 induction and AMPK activation might be regulated by distinct pathways and converge to promote the activation of the TSC1/2 complex (Sofer, Lei et al. 2005). To clarify these ambiguities, we tested the effect of chronic energy depletion on mTOR Complex1 signaling in AMPK α_1/α_2- deficient MEFs. The result of this experiment showed that the chronic energy depletion induced by 2DG inhibited mTOR Complex1 signaling regardless of AMPK status (Figure 25). Although, we do not observe the chronic response at 4 hours, as reported previously (Sofer, Lei et al. 2005), it is evident at 8 hours in our case (Figure 25). We were unable to verify the onset of the chronic response with the induction of REDD1 protein during this time course due to immuno-detection sensitivity issues (Ellisen, Ramsayer et al. 2002). Moreover, that AMPK was not involved in inducing inhibition of mTOR Complex1 signaling during chronic energy deprivation was also

verified with a more physiologically relevant energy deprivation condition, glucose starvation (Figure 26). Taken together, these observations clearly demonstrate that AMPK is not required for the regulation of mTOR Complex1 by chronic energy deprivation.

4. Possible mechanism involved in the acute energy deprivation response to mTOR Complex1 signaling

mTOR Complex1 is composed of mTOR plus its two binding partners raptor and mLST8. Although initial knockdown studies showed that both partners were required for mTOR Complex1 signaling, recent deletion in mice revealed that only raptor was essential for mTOR Complex1 activity (Guertin, Stevens et al. 2006). mTOR-raptor interaction correlates well with its in-vitro kinase activity and both have been shown to be sensitive to the action of 2DG and to the mitochondrial electron transport inhibitor, antimycin A (Kim, Sarbassov et al. 2002). PRAS40, a new component of the mTOR Complex1, has been reported to be phosphorylated by PKB/Akt and transduces the insulin signal to mTOR Complex1 by relieving its suppressive effects on mTOR Complex1 (Sancak, Thoreen et al. 2007; Vander Haar, Lee et al. 2007). This new component is thought to integrate insulin signaling as well as nutrient signaling since deprivation of either factor increases phosphorylation of PRAS40 and decreases its interaction with mTOR Complex1. However, the role of PRAS40 in mediating energy deprivation and mitochondrial metabolic stress signals to mTOR Complex1 is not clear, since one group observes a correlation between these stresses and an increase in PRAS40 affinity for mTOR, which would translate into a decrease in mTOR Complex1 activity (Vander Haar, Lee et al. 2007). On the other hand the second group does not see changes upon energy depletion induced signals to mTOR Complex1 as measured in an in vitro mTOR Complex1 kinase assay (David Sabatini, personal communication) (Sancak, Thoreen et al. 2007). Nevertheless, both groups claim a level of regulation of mTOR Complex1 by PRAS40, which would by-pass the need for TSC1/2 complex. Thus PRAS40 appears as quite an attractive model for mTOR Complex1 regulation by energy deprivation since our studies revealed that contrary to what is currently thought (Inoki,

Zhu et al. 2003), mTOR Complex1 does not require the TSC1/2 complex for its regulation by energy levels (Figures 12-14)

In line with possible regulators of mTOR Complex1 by energy deprivation, our laboratory has recently described a role for class 3 PI3-kinase, hVps34, in the regulation of mTOR Complex1 by nutrients (Nobukuni, Joaquin et al. 2005). hVps34 is an upstream activator of mTOR Complex1 signaling, which has been shown to mediate the amino acid signals to mTOR Complex1 independently of the TSC1/2 complex. Moreover, its activity has been found to be regulated by glucose deprivation and oligomycin treatment (Byfield, Murray et al. 2005). Since hVps34 was proposed to act as a primary nutrient and energy sensor, we tested whether hVps34 could mediate energy deprivations signals to mTOR Complex1. Firstly, hVps34 knock-downs by RNA interference prevented the stimulation of S6K1 T389 phosphorylation by glucose (data not shown). Next we measured hVps34 activity upon 2DG treatment and glucose starvation and unexpectedly found that there was no change in hVps34 in vitro activity (Figure 30). At present, we cannot explain the discrepancies between our studies and those published by Byfield et al. (Byfield, Murray et al. 2005). Based on our findings, hVps34 would have a permissive role in mTOR Complex1 signaling.

Reactive oxygen species (ROS) are by-products of mitochondrial metabolism. Since our studies have shown that mTOR Complex1 is sensitive to mitochondrial metabolism, it is possible that ROS might serve as a direct signaling molecule in regulating mTOR Complex1 signaling. Energy dependent mTOR Complex1 functions, such as ribosome biogenesis, could therefore adjust directly to the rate of mitochondrial metabolic activity, indicative of the rate of respiration and ATP production, by coupling it directly to a “side” product of its metabolism. Interestingly, hydrogen peroxide one of the many intermediates of ROS metabolism has been shown to affect mTOR Complex1 signaling. Although hydrogen peroxide was originally shown to activate S6K1 T389 phosphorylation (Bae, Seo et al. 1999), more recent studies indicated that hydrogen peroxide would enhance the mTOR-raptor interaction and therefore inhibit downstream phosphorylation of S6K1 (Kim, Sarbassov et al. 2002). Despite the controversy surrounding the role of ROS in mTOR Complex1 signaling, the mTOR-raptor complex has been shown to be sensitive to a redox mechanism (Sarbassov and Sabatini 2005). The authors speculated that mTOR itself could serve as a redox sensor which would regulate its interaction with raptor and its downstream effectors. Indeed, the presence of conserved cysteine residues within the highly conserved FATC domain of mTOR could

provide a mechanism by which mTOR could regulate its activity through a redox dependent change in conformation (Dames, Mulet et al. 2005). We addressed this possibility by testing the effects of the oxidant PAO on mTOR Complex1 signaling. Our observations showed that PAO treatment increased basal levels of S6K1 T389 phosphorylation. However, 2DG still induced S6K1 T389 dephosphorylation to the same extent as non-PAO treated cells. We therefore do not think that oxidation by PAO protects mTOR Complex1 from the effects of 2DG. In contrast, Sarbassov et al. concluded that a redox-based signaling mechanism operates to regulate mTOR Complex1 in response to energy depletion. However, closer inspection of their data shows similar results to ours, that is, PAO increased basal levels of S6K1 T389 dephosphorylation, but did not protect against 2DG treatment (Sarbassov and Sabatini 2005).

As explained earlier, we also observe clear changes in $\Delta\Psi_m$ with 2DG and oligomycin treatment. This observation raises the possibility as to whether $\Delta\Psi_m$ is a factor required for ATP's effect on mTOR Complex1 or whether $\Delta\Psi_m$ might play a direct role in signaling to mTOR Complex1. An experiment that could address this issue is to abolish oxidative phosphorylation in the cell and assess whether mTOR Complex1 is sensitive to changes in $\Delta\Psi_m$. For this purpose, it would be interesting to test ATP depleting agents in mitochondria DNA-less cells (mtDNA-less or ρ^0 cells). ρ^0 cells lack the genetic information encoded by mtDNA for 13 essential subunits of the respiratory chain including the mitochondrial ATP synthetase. This deficiency makes these cells entirely dependent on glycolysis for their energy source as they are unable to generate ATP by respiration. Nevertheless, these cells have been shown to retain $\Delta\Psi_m$. despite that no respiration is operating. In this context, one could test whether $\Delta\Psi_m$ on its own can affect mTOR Complex1 signaling or assess the effect of the ATP depleting agents in this situation. Testing 2DG in the absence of electron transport chain would help elucidate the role of $\Delta\Psi_m$ in the energy deprivation signal to mTOR Compex1 and would also address whether $\Delta\Psi_m$ can be decoupled from ATP's role in regulating mTOR Complex1. In relation to $\Delta\Psi_m$, it should be noted that the oligomycin-mediated response to mTOR Complex1 is transient as S6K1 T389 phosphorylation started to recover by 1 hour (Figure 13). We reported this phenomenon as the cell's switch to an alternative energy source. The implication of $\Delta\Psi_m$ in the recovery response and this energy source switch could be tested with the use of uncoupling agents or "proton translocators". These

agents include a number of weak organic acids such as carbonylcyanide – m – chlorophenylhydrazone (CCCP) which can be protonated and due to their lipohilic properties can cross the mitochondria bilayer membrane and reach the matrix where they can be deprotonated. These properties allow them to effectively abolish $\Delta\Psi_m$ by short circuiting and dissipating the proton motive force necessary for the ATP production by the ATP synthetase. Treatment of cells with CCCP during the recovery of the mTOR Complex1 signaling could highlight the importance of $\Delta\Psi_m$ for this energy switch. In that respect it is interesting to note that mTOR has been shown to be localized at the outer membrane of the mitochondria (Desai, Myers et al. 2002). This "retrograde signaling" would serve as a checkpoint for mTOR Complex1, such that it would monitor the availability of its resources in the cell. It has already been shown that metabolic intermediates or entities of mitochondria can serve as a signaling elements in the cell, as is the case for succinate, an intermediate of the TCA cycle (Selak, Armour et al. 2005) and the well described resident mitochondrial protein cytochrome C, which upon its release triggers apoptosis (Li, Nijhawan et al. 1997). Interestingly, evidence exists that the branched-chain amino acid leucine indirectly activates mTOR Complex1 signaling through mitochondrial metabolites, demonstrating a link between mTOR Complex1 and mitochondrial metabolism (Xu, Kwon et al. 2001). Further studies at elucidating the significance of mTOR localization at the mitochondria would shed some light as to the relationship between mTOR and mitochondria in the context of nutrient and energy regulation. In conclusion, our studies showed that $\Delta\Psi_m$ did vary with both 2DG and oligomycin and could potentially be an important factor to consider in future studies for the understanding of the regulation of mTOR Complex1 by cellular energy.

5. Metformin and cancer: role of mTOR Complex1 signaling

Metformin is the most widely prescribed drug for the treatment of *diabetes mellitus* type II. Its therapeutic properties have been linked to lowering hyperglycemia through the inhibition of hepatic gluconeogenesis and increasing glucose uptake by skeletal muscle (Zhou, Myers et al. 2001). These physiological benefits have been attributed to the activation of AMPK, which has been supported by studies carried out in LKB1 knock-out animals (Shaw, Lamia et al. 2005). However, recent studies have questioned the role of AMPK in inhibiting glucose phosphorylation and glycolysis in the liver since metformin could still affect these processes in the absence of AMPK as seen in AMPK α_1/α_2- deficient hepatocytes (Guigas, Bertrand et al. 2006). With respect to glucose uptake in skeletal muscle, both mTOR Complex1 and S6K1 have been implicated in this increase through their ability to suppress insulin signaling via a negative feedback loop from S6K1 to IRS1 (Um, D'Alessio et al. 2006). These effects are thought to be largely mediated by the phosphorylation of IRS1 at sites which either disrupt its interaction with the insulin receptor or antagonize PI3K binding (Um, D'Alessio et al. 2006). Moreover, recent studies showed that both metformin and 2DG reverse the mTOR Complex1 mediated inhibition of PI3K by decreasing mTOR Complex1 phosphorylation of IRS1 (Tzatsos and Kandror 2006). The authors concluded that these effects were mediated by the LKB1-AMPK-TSC2 axis to inhibit mTOR Complex1, since AMPK T172 phosphorylation was increased by both agents (Tzatsos and Kandror 2006). However, our findings argue that metformin inhibits mTOR Complex1 via an AMPK-independent pathway (Figure 27A). This effect was also observed with the phenyl analog, phenformin (Figure 27B). Importantly, that TSC2 is not required to elicit these inhibitory responses strongly suggests that the inhibitory effects observed with metformin and phenformin operate on the same pathway as those employed by 2DG and oligomycin for inhibition of mTOR Complex1 signaling (Figure 28 and see model at Figure 29). Taken together, our studies reveal the existence of an AMPK-TSC2 independent pathway which regulates mTOR Complex1 in response to rapid changes in cellular energy status. Although it is not fully clear whether metformin induces a change in the AMP:ATP ratio (Hawley, Gadalla et al. 2002) (Hardie 2006) (Hoek), metformin seems to act on the same pathway to affect mTOR Complex1 signaling as the one revealed here in our studies of acute energy deprivation. Moreover, the importance of this autonomous energy dependent mTOR Complex1 signaling pathway is underscored

by the use of metformin and 2DG in the clinic for the treatment of diabetes (Evans, Ogston et al. 2006) and cancer (Maher, Wangpaichitr et al. 2007). Therefore the elucidation of the molecular mechanism by which AMPK-TSC2 independent pathway regulates mTOR Complex1 signaling can have a clear impact on the treatment of specific human pathologies, including Tuberous Sclerosis and Lymphangioleiomyomatosis.

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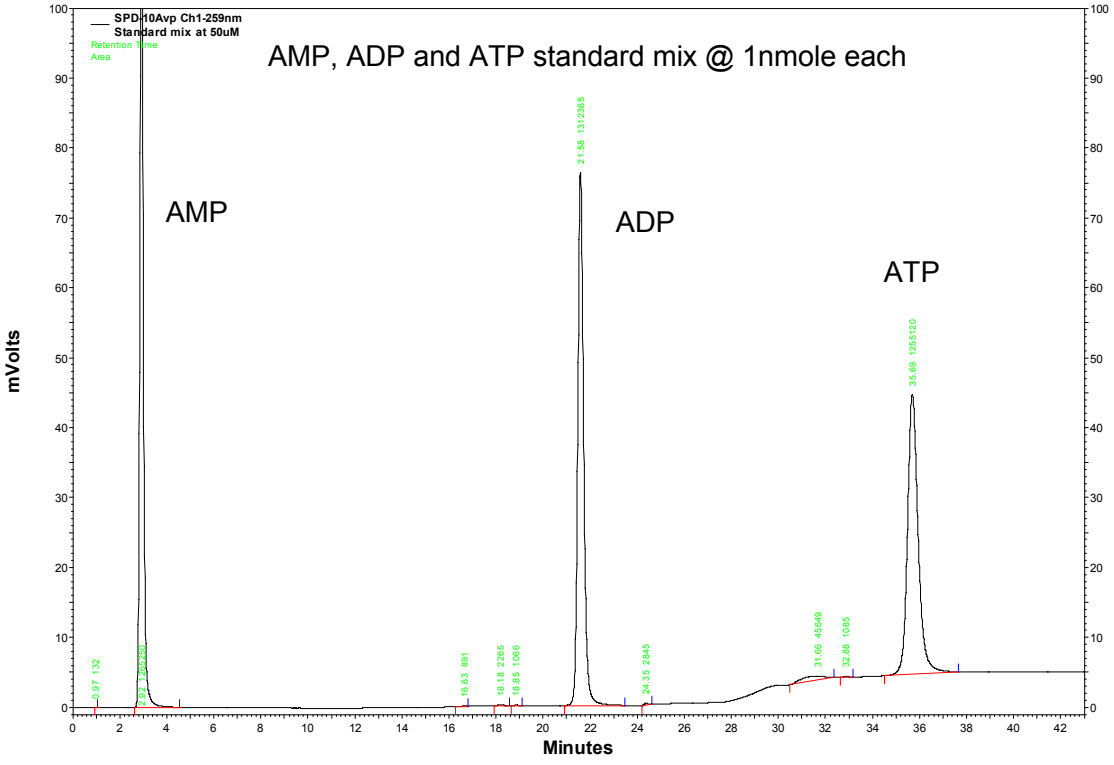
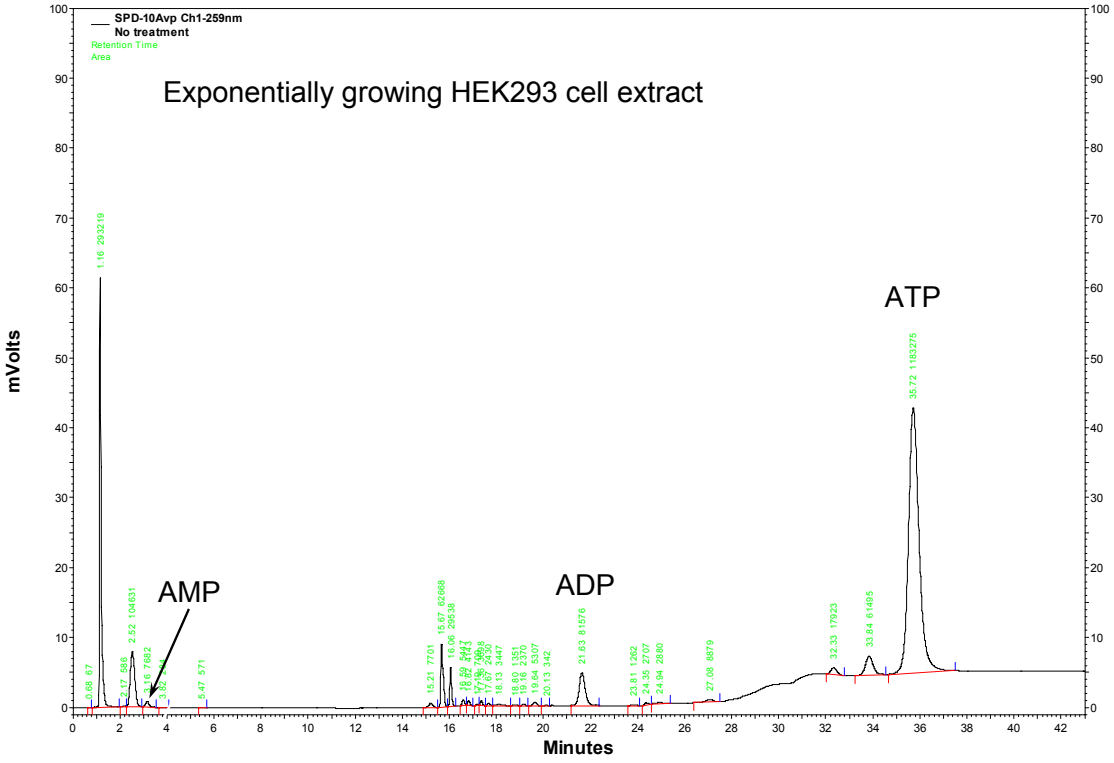
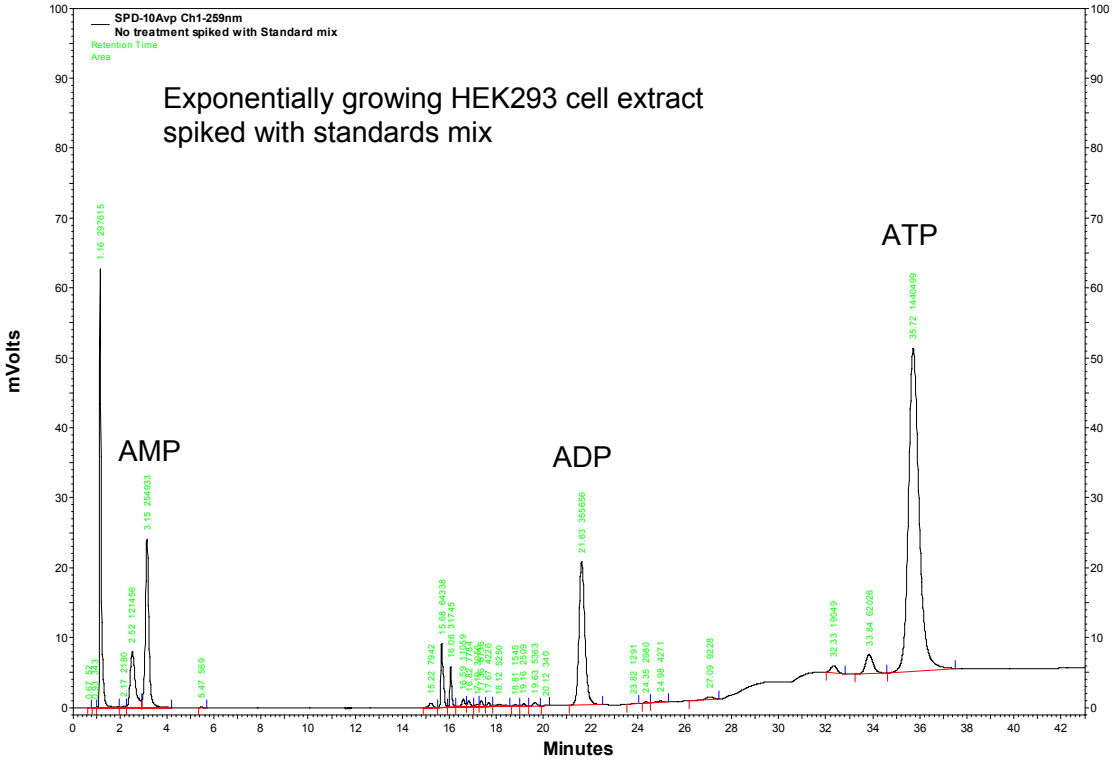
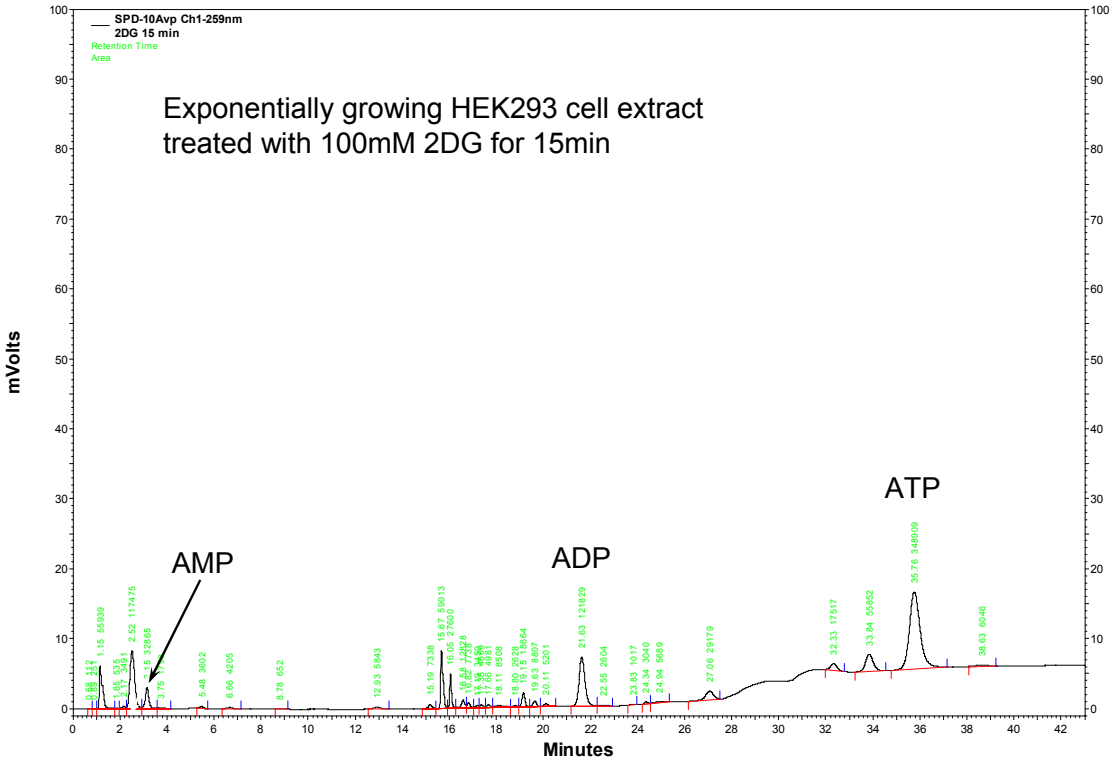
VII. APPENDIXES

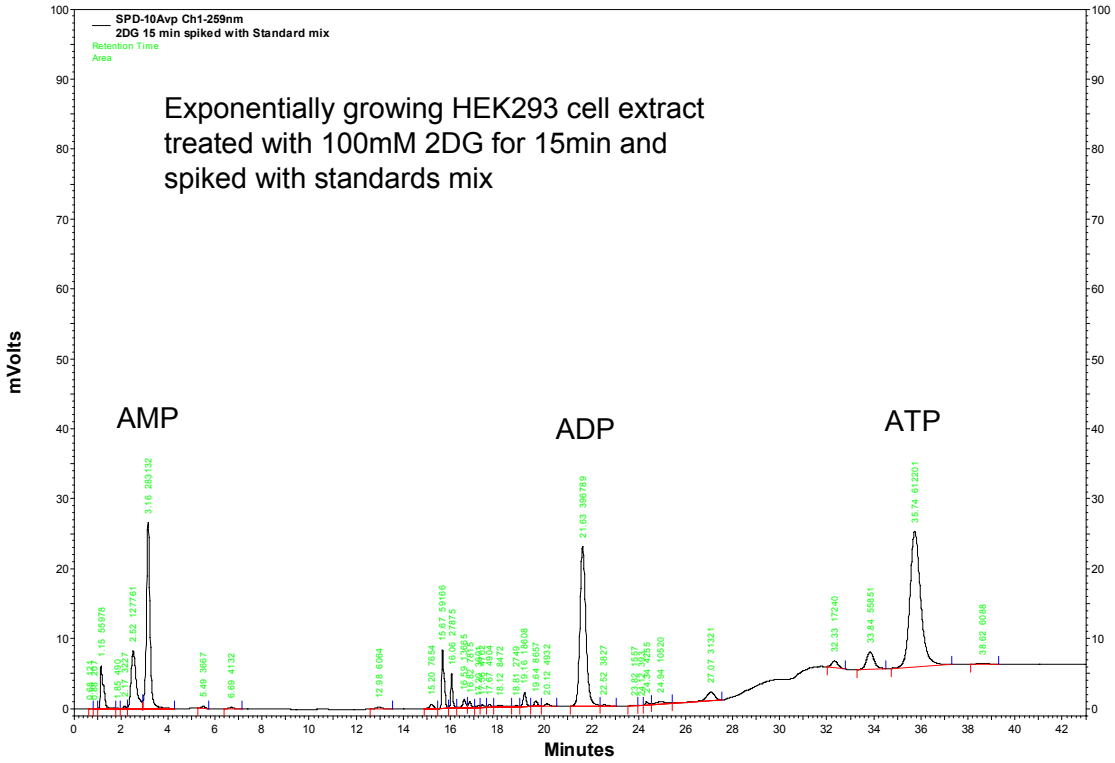
APPENDIX 1: Representative chromatograms of adenine nucleotide measurements by HPLC. AMP, ADP and ATP pics are indicated.



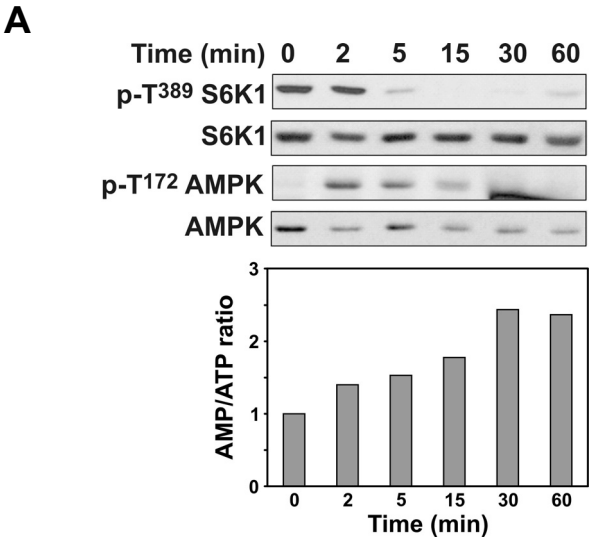


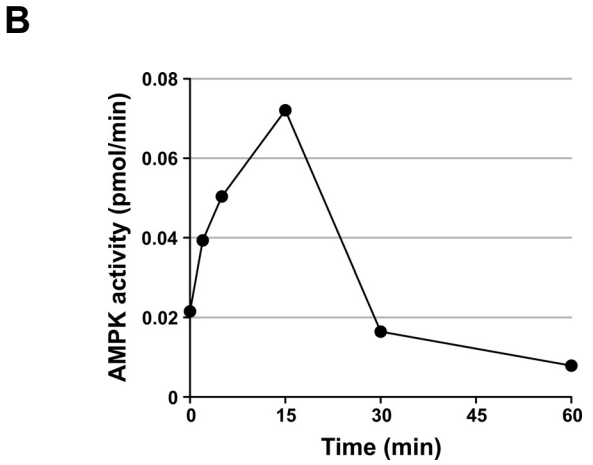

SPD-10Avp Ch1-259nm
No treatment spiked with Standard mix
Retention Time
Area
Exponentially growing HEK293 cell extract
spiked with standards mix
AMP
ADP
ATP
mVolts
Minutes


SPD-10Avp Ch1-259nm
2DG 15 min
Retention Time
Area
Exponentially growing HEK293 cell extract
treated with 100mM 2DG for 15min
AMP
ADP
ATP
mVolts
Minutes


SPD-10Avp Ch1-259nm
2DG 15 min spiked with Standard mix
Retention Time
Area
Exponentially growing HEK293 cell extract
treated with 100mM 2DG for 15min and
spiked with standards mix
AMP
ADP
ATP
mVolts
Minutes

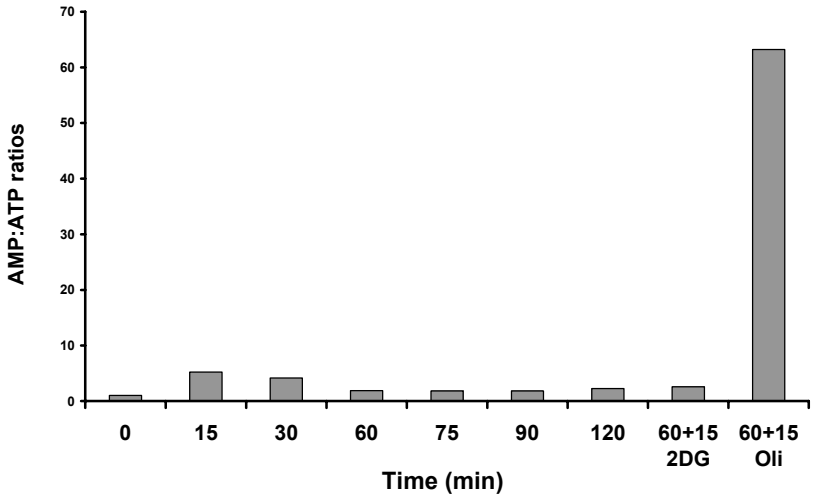
APPENDIX 2: Exponentially growing A549 cells treated with 100 mM 2DG for the times indicated. Parallel plates were either extracted for Western blotting or for the measurement of ATP by luminometry. **B)** Analysis of the *in vitro* kinase activity of AMPK from exponentially growing A549 cells treated as in **A)**.



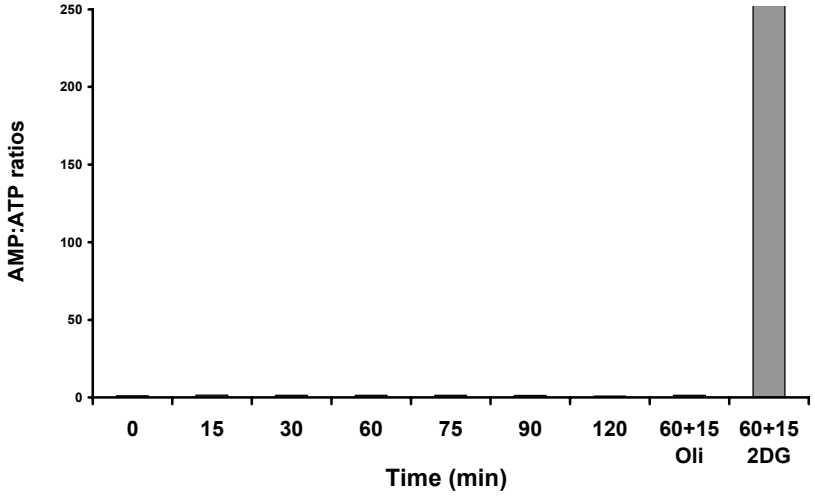


APPENDIX 3: AMP:ATP ratios of recovery experiments with **A)** 2DG and **B)** oligomycin. See Figures 15 A and B respectively.

A



B



APPENDIX 4: Manufacturers and catalogue numbers of reagents used

2-deoxy-α-D-glucose: Fluka/Sigma-Aldrich #31060

4-20% Tris-HCl Criterion SDS PAGE gel: Bio-Rad #345-0033

4E-BP1 antibody: Santa Cruz Biotechnology #sc-6936

β-actin antibody: Cell Signaling Technology #4967

ATP bioluminescence Assay Kit CLS II: Roche Diagnostics #1 699 695

Anion exchange column for adenine nucleotide measurements by HPLC: WVS Partisphere 5 SAX, 4.6X125mm Whatman #4621-0505

Anti-goat HRP antibody: DakoCytomation #P0160

Anti-mouse HRP antibody: GE Healthcare Life Sciences #NA931V

Anti-rabbit HRP antibody: GE Healthcare Life Sciences #NA934V

ACC phospho-S79 antibody: Cell Signaling Technology #3661

AICAR (5-Aminoimidazole-4-carboxamide-1- β-D-ribofuranoside): Toronto Research Chemicals # A611700

AMPK phospho-T172 antibody: Cell Signaling Technology #2535

AMPK α1,α2 antibody: Cell Signaling Technology #2532

BCA protein assay kit: Pierce Biotechnology #23227

Blocker for Western Blots: Bio-Rad #170-6404

Chromatography paper for semi-dry protein transfer: Whatman 17 CHR #3017-915

DMEM High Glucose: Hyclone #SH30243.01

ECL reagents: Pierce Biotechnology #34080

Fetal Bovine Serum: Hyclone #SH30088.03

Hexokinase II antibody: Santa Cruz Biotechnology #sc-6521

Insulin: Sigma-Aldrich #I0516

Metformin (1,1-Dimethylbiguanide hydrochloride): Sigma-Aldrich #D5035

Oligomycin: Acros Organics #230350250 and Calbiochem #495455

PBS 10X: Fisher Scientific #BP399-1

Penicillin/Streptomycin 100x mix: Mediatech/Cellgro # 30-001-CI

Phenformin (Phenethylbiguanide): Sigma-Aldrich #P7045

Protease inhibitor cocktail (Complete, EDTA-free): Roche Diagnostics #11 873 580 001

PVDF membrane: Millipore #IPVH00010

S6K phospho-T389 antibody: Cell Signaling Technology #9206

S6K antibody: Santa Cruz Biotechnology #sc-230

TBS 10X: Fisher Scientific #BP2471-1

Trypsin 0.25%: Hyclone #SH30042.01

TSC2 antibody: Cell Signaling Technology #3612

Tubulin antibody: Monoclonal antibody #E7 was developed by Michael Klymkowsky and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by The University of Iowa. http://dshb.biology.uiowa.edu/ccp1344-tubulin-28beta-29-e7.htm

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ADEM KALENDER

SUMMARY OF QUALIFICATIONS

- Master of Science in Industrial Biotechnology
- Bachelor of Science in Biomedical Technology
- Laboratory Assistant in Medical Biology

EDUCATION

2002–2007 *University of Basel*, Switzerland, PhD degree in Biochemistry pursued at the *Friedrich Miescher Institute for Biomedical Research* of the *Novartis Research Foundation* and from October 2004 at the *Cancer and Cell Biology Department* of the *Genome Research Institute, University of Cincinnati*, USA

1996–1997 *University of Newcastle upon Tyne*, United Kingdom, Master of Science in Industrial Biotechnology. MSc thesis performed at *SmithKline Beecham Pharmaceuticals*, United Kingdom (April-October 1997) entitled: "Molecular Cloning of genes of a basidiomycete strain"

1993–1995 *Catholic University of Louvain*, Belgium, Bachelor of Science in Biomedical Technology. BSc research thesis performed at the *Ludwig Institute for Cancer Research*, Belgium (September 1994-August 1995) entitled: "The gene RAGE coding for an antigen recognized by autologous CTL on a human renal carcinoma: Search for the complete cDNA and the antigenic peptide" (Immunogenetics. 1996;44(5):323-30)

1988–1992 *De-Mot Couvreur Institute*, Belgium, Laboratory Assistant diploma in Medical Biology and Cytology. Diploma project performed at the National Institute for Veterinary Research, Belgium (July 1991) entitled: "Development of diagnostic approaches for the detection of the Bovine Torovirus"

1988 *Athénée Royale de Schaerbeek*, Belgium, Secondary School Diploma

PUBLICATIONS

"Metformin inhibits mTOR Complex 1 through an autonomous energy dependent pathway, independent of TSC1/2 and AMPK". In revision for the journal Cell Metabolism

"mTOR, energy and cancer". Review article in preparation for the journal Cancer Research.

PROFESSIONAL EXPERIENCE

November 2007 – present: *Cancer and Cell Biology Department, Genome Research Institute*, *University of Cincinnati*, Cincinnati, USA. Postdoctoral fellow, involved in a research project investigating the regulation of mTOR/S6K1 signaling pathway by cellular energy.

April 1998 – September 2001: *GlaxoSmithKline Pharmaceuticals*, Harlow, United Kingdom. Associate Scientist at the Cell Signaling group of the Molecular Neurobiology Department. In charge of developing secondary assays for the screening of analog compounds coming from SAR and of exploratory studies aimed at deciphering the signaling pathway involved in chronic pain by isolating substrates of a candidate kinase (Neuroscience. 2000;101(3):767-77)

January – April 1998: *SmithKline Beecham Pharmaceuticals*, Worthing, United Kingdom. Screening Scientist at the Biotechnological Development Unit. In charge of developing a fungal strain producing a candidate antibiotic effective on penicillin and streptomycin resistant strains

August 1994, July 1992, 1990 – 1991: *Erasmus and Brugmann Hospitals*, Belgium. Technician at the Departments of Bacteriology and Transfusion

AWARDS & FELLOWSHIP RECEIVED

-Postdoctoral fellowship by an appointment to the Research Participation Program at the Air Force Research Laboratory, Human Effectiveness Directorate, Bioscience and Protection, Wright Patterson AFB administered by the Oak Ridge Institute for Science and Education, USA 2007-2009

-Krebsliga Award from the Swiss National Cancer League for the PhD degree at the University of Basel, Switzerland, 2001-2004

-British Council Award for the Master of Science in Industrial Biotechnology at the University of Newcastle upon Tyne , United Kingdom, 1996-1997

SELECTED COURSES AND SCIENTIFIC MEETINGS ATTENDED

The LAM Foundation/NHLBI, USA, 2005-2009: International Research Conference on Lymphangioleiomyomatosis. Poster presented.

EMBO conference on Cellular Signaling, Croatia, June 2003: International meeting on Cellular Signaling and Molecular Medicine. Poster presented.

NATO/FEBS Advance Study Institute, France, September 2000: International Summer School on Protein Modules in Cellular Signaling. Poster presented.

Cold Spring Harbor Laboratory, USA, June 1999: Course on Molecular Mechanisms of Neurodegenerative Diseases. Oral presentation at workshop.

The European Neuroscience Symposium, Germany, June 1998: International Research Conference in neurosciences